

## VACCINE AND COMPOSITIONS FOR THE PREVENTION AND TREATMENT OF NEISSERIAL INFECTIONS

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The invention was made with the support of NIH Grant No. 5UI9 AI43924 and AI 43924-05. The U.S. government has certain rights in the invention.

### Claim of Priority

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This application is a continuation-in-part application of U.S. Serial No. 10/066,551, filed January 31, 2002, which claims benefit under 35 U.S.C. 119(e) from U.S. Provisional Application Serial No. 60/266,070, filed January 31, 2001; U.S. Provisional Application Serial No. 60/310,356, filed August 6, 2001; and U.S. Provisional Application Serial No. 60/344,452, filed October 23, 2001; all of which  
15 are incorporated herein by reference.

### Background of the Invention

*Neisseria gonorrhoeae* is the causative agent of the disease gonorrhea. Approximately 300,000 women a year contract gonorrhea in the U.S. Worldwide,  
20 the number of women with this infection is in the millions. It is a major cause of infertility and pelvic inflammatory disease. It is also a major co-factor in the spread of HIV1.

In men, gonococcal infection develops as an acute urethritis that is typically characterized by a purulent discharge that results as a consequence of the concurrent  
25 inflammatory response to infection. In women, gonococcal infection can develop as an ascending infection of the genital tract that can lead to an acute pelvic inflammatory disease, infertility, or ectopic pregnancy. High proportions of women, however, initially develop asymptomatic gonococcal infections, in contrast to *N. gonorrhoeae* infection in men.

The mechanisms by which the gonococcus infects and invades the female genital tract are only beginning to be understood. Research has shown that gonococci are capable of invading primary human epithelial cells derived from both the endo- and the ectocervix. These studies implied that the mechanism(s) used by the gonococcus to breach the cervical epithelium are distinct from those mechanisms used to invade the urethral epithelium of men and that several endocytic mechanisms appear to play a role in gonococcal invasion of the female genital tract.

*Neisseria meningitidis* is one of the leading causes of bacterial meningitis worldwide, affecting mainly children and young adults. The genomic sequence of *Neisseria meningitidis* B and *Neisseria meningitidis* A has been published (see, for example, Tettelin et al., Science, 287, 1809-1815 (2000) and Parkhill et al., Nature, 404, 502-506 (2000), respectively). The rapid progression of meningococcal disease makes proper diagnosis and subsequent treatment often vital to the survival of infected individuals. If not properly diagnosed and treated, meningococcal infections can lead to shock and death within a matter of hours. Thus, better prevention, diagnosis and treatment of meningococcal infections would be invaluable.

Currently there is no vaccine for the prevention of gonorrhea or for the treatment of meningococcal meningitis. Therefore, there is a need for an effective means to prevent or ameliorate neisserial infections.

### **Summary of the Invention**

The present invention provides a polypeptide, polynucleotide, vaccine, and a method of vaccination effective to immunize a mammal against a neisserial infection, *e.g.*, an infection caused by *Neisseria gonorrhoeae* or *Neisseria meningitidis*. Such immunization can prevent, ameliorate or reduce the incidence of gonorrhea and/or meningococcal infection in a human. The vaccine contains an immunogenic amount of a neisserial phospholipase D (PLD) polypeptide in combination with a physiologically-acceptable, non-toxic vehicle. Examples of

neisserial PLD include gonococcal PLDs, such as SEQ ID NO:4, SEQ ID NO:14, SEQ ID NO:16 and SEQ ID NO:18, and meningococcal PLD such as SEQ ID NO:20.

In addition, the invention provides a transgenic *Neisseria* bacterium comprising a disrupted *pld* gene wherein the bacterium has reduced phospholipase D activity as compared to the phospholipase D activity of a corresponding wild-type *Neisseria*. In one embodiment of the invention, the transgenic *Neisseria* bacterium is *N. gonorrhoeae*, e.g., *N. gonorrhoeae* strain 1291, *N. gonorrhoeae* strain FA1090, or *N. gonorrhoeae* strain MS11. In another embodiment of the invention, the transgenic *Neisseria* bacterium is *N. meningitidis*, e.g., a *N. meningitidis* encapsulated strain or a *N. meningitidis* acapsular mutant strain.

The *pld* gene of a transgenic bacterium of the invention can be disrupted by mutagenesis, for example, by insertion mutagenesis, deletion mutagenesis, substitution mutagenesis, or a combination thereof. Such a transgenic bacterium may have reduced amounts of phosphatidic acid and choline as compared to a corresponding wild-type *Neisseria*. In one embodiment of the invention, the transgenic bacterium has reduced toxicity as compared to a corresponding wild-type *Neisseria*.

As an example, the *pld* gene of the transgenic bacterium comprises a nucleic acid sequence such as SEQ ID NO:9, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17 or SEQ ID NO:19.

Also provided herein is an isolated and purified polynucleotide comprising a *pld* gene from a *Neisseria* bacterium. In one embodiment, the polynucleotide is a *N. gonorrhoeae* sequence, e.g., SEQ ID NO:9, SEQ ID NO:13, SEQ ID NO:15 or SEQ ID NO:17. In another embodiment, the polynucleotide is a *N. meningitidis* sequence, e.g., SEQ ID NO:19.

The invention provides an isolated and purified polypeptide encoded by a nucleic acid sequence that is SEQ ID NO:9, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17 or SEQ ID NO:19.

In addition, the invention provides an isolated and purified polypeptide comprising phospholipase D from a *Neisseria* bacterium, such as polypeptides including SEQ ID NO:4, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18 or SEQ ID NO:20.

5           The invention provides a vaccine comprising an immunogenic amount of a PLD polypeptide from *Neisseria*, for example, a polypeptide encoded by SEQ ID NO:9, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17 or SEQ ID NO:19, which amount is effective to immunize a patient against a neisserial infection, in combination with a physiologically-acceptable, non-toxic vehicle. A vaccine of the  
10   invention may also include an effective amount of an immunological adjuvant. In one embodiment, the PLD polypeptide is conjugated or linked to a second peptide. In another embodiment, the PLD polypeptide is conjugated or linked to a polysaccharide.

          The invention also provides a method of protecting a patient against  
15   *Neisseria* colonization or infection, for example, a *Neisseria gonorrhoeae* and/or *Neisseria meningitidis* colonization or infection, comprising administering to the patient an effective amount of a vaccine comprising an immunogenic amount of a PLD polypeptide from *Neisseria*, which amount is effective to immunize a susceptible patient against a neisserial infection, in combination with a  
20   physiologically-acceptable, non-toxic vehicle. For example, the PLD polypeptide may be encoded by a polynucleotide comprising SEQ ID NO:9, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17 or SEQ ID NO:19. Such a vaccine may also contain an immunological adjuvant. The PLD can also be conjugated or linked to a second peptide or a polysaccharide. The vaccine can be administered orally,  
25   mucosally or by subcutaneous or intramuscular injection.

          Also provided is a method of preventing infection or colonization of *Neisseria* in a patient, *e.g.*, *Neisseria gonorrhoeae* and/or *Neisseria meningitidis*, by administering to the patient a compound that inhibits bacterial phospholipase D,

*e.g.*, neisserial PLD. In one embodiment, the compound is an antibody specific for a neisserial PLD.

Also provided is an anti-neisserial PLD antibody.

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### **Brief Description of the Figures**

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

**FIG. 1.** Anti-CR3 antibodies inhibit gonococcal attachment and invasion of  
10 CR3-transfected CHO cells. CHO-CR3 cell monolayers were pretreated with antibodies to the CR3 alpha subunit CD11b (H5A4, Bear1) or CD18 (anti-CD18, IB4) or anti-CD18 antibody and an anti-CD18 blocking peptide as outlined in the text. Antibody-treated cell monolayers were challenged for two hours with gonococci prior to gentamicin addition, cervical cell lysis, and plating of serial  
15 dilutions to determine colony-forming units. Gentamicin treatment was omitted from attachment assays. Percent inhibition was determined as a normalized function of the ability of gonococci to adhere to or invade CHO-CR3 cells in the absence of antibody. Values given are the mean values of at least three trials. A Kruskal-Wallis analysis of variance was used to determine the statistical significance of the  
20 invasion assays.  $p$ -values were less than 0.05 for all of the antibodies tested alone.

**FIG. 2.** Anti-CR3 antibodies inhibit gonococcal invasion of primary cervical cells. Invasion of primary, human, ecto- and endocervical cell monolayers by gonococci was performed as outlined in the text. Antibodies H5A4 and Bear1 recognize the CR3 alpha subunit, CD11b. Anti-CD18 and IB4 are specific for the  
25 CR3 beta subunit, CD18. Inhibition of invasion was determined as a normalized function of the ability of gonococci to invade primary ecto- and endocervical cells in the absence of antibody. Values given are the mean values of at least three trials. A Kruskal-Wallis analysis of variance was used to determine the statistical

significance of the invasion assays.  $p$ -values were less than 0.05 for all of the antibodies tested.

**FIG. 3.** The effect of *Clostridium* C3 neurotoxin on the invasion of primary cervical cells by *N. gonorrhoeae*. The ability of gonococci to invade primary ecto- and endocervical cells was determined as outlined in the text. Invasion was determined as a proportion of the original infection inoculum. Values given are the mean values of three trials. A Kruskal-Wallis analysis of variance was used to determine the statistical significance of percent invasion with C3 toxin in comparison to percent invasion without C3 toxin treatment.  $p$ -values were 0.05 for each assay.

**FIG. 4.** Laser scanning confocal microscopy (LSCM) demonstrates that the characteristic cytokeratin staining pattern of the tissue biopsies has been retained in the respective primary cervical epithelial cell cultures. Sectioned tissue biopsies and primary cervical epithelial cell monolayers were incubated with a FITC-conjugated antibody to the noted specific cytokeratin. Ethidium bromide was used to counter-stain the tissue sections. Endocervical cells labeled intensely with antibody 8.12, which is specific for type I cytokeratins 13, 15, and 16 (panel A). The labeling of endocervical cells with an antibody specific for type II cytokeratin 4 (panel C) was considerably less intense, and it was not uniformly distributed. Ectocervical cells labeled positive with an antibody specific for cytokeratins 13, 15, and 16 (panel B) and cytokeratin 4 (panel D).

**FIG. 5.** Scanning electron microscopy (SEM) analysis shows the predominant changes that occur in the cervical cell membrane over the course of a three hour infection as the result of cervical epithelial cell-gonococcal interactions. At early (0 to 60 minutes) phases of infection *N. gonorrhoeae* could be found on the surface of endocervical cells either associated with microvilli (A) or undergoing an endocytic process (B). As the infection process continued, microvilli appeared to acquire directionality. Filopodia/lamellipodia became evident after thirty minutes post infection (C). Bacteria appeared to be in the process of internalization (D).

Loss of microvilli with a smoothing of the cervical cell membrane around the periphery of some sites of gonococcal infection also became evident at approximately thirty minutes post infection (E). Membrane ruffles (F, endocervical; G, ectocervical) appeared at sixty minutes, and they became prevalent at ninety minutes post infection. Ruffles could be induced to occur at approximately thirty minutes post infection with use of a primed infection inoculum (see Example 1) (G). A visible smoothing of the cervical cell membrane encircling membrane ruffles can be seen (H). By three hours post infection large ruffles could readily be observed.

**FIG. 6.** Bright-field light microscopy (BFLM) and immuno-transmission electron microscopy (TEM) studies demonstrate ruffling of the cervical surface and invasion of the primary cervical epithelial cells at ninety minutes and three hours post infection. For TEM analysis, bacteria were labeled with an antibody specific to the gonococcal surface protein, H.8; cervical cells were labeled with a polyclonal antibody to actin. 30 nm and 10 nm gold bead antibody-conjugates were used to label the bacteria- and host-specific primary antibodies, respectively. Membrane protrusions can be seen that are labeled with actin and that are encompassing gonococci at ninety minutes after the onset of infection (A). Bacteria can also be seen entering the cervical cell as individual entities in actin-lined, spacious vacuoles (B). Large membrane ruffles can be seen associating with gonococci at three hours post infection C and D). For BFLM (D) thick (1  $\mu$ m) paraffin sections of endocervical cells were stained with hematoxylin and eosin. Arrows denote bacteria. Bar = 2  $\mu$ m.

**FIG. 7.** This figure shows TEM studies of cervical biopsies from women with gonococcal cervicitis. Panels A and B demonstrate cytoskeletal changes and membrane ruffling occur during naturally acquired gonococcal infection. Panel A and panel B show large and small membrane protrusions associated with gonococci (designated by arrows) that are similar to those seen in figure 6.

**FIG. 8.** Differential interference contrast (DIC) and LSCM analysis demonstrate co-localization of *N. gonorrhoeae* 1291-green with a concentrated

accumulation of the actin-associated protein, vinculin. In panel A, vinculin was immunolabeled with a TRITC-conjugated antibody and in a colored version of this figure was visible as a red fluorescence (A); in panel B, bacteria were transformed with green fluorescent protein (GFP) and in a colored version of this figure were visible as a green fluorescence. (C) In a merged image of panels A and B, arrows denote co-localization of bacteria with vinculin, which was visualized in a colored version of this figure as a yellow-orange because of the combined signal of the individual fluorophores. (D) Merged LSCM and DIC image (of the ectocervical cells). Similar results were seen with endocervical cells and for the actin-associated proteins ezrin and myosin, but the focal accumulation of  $\alpha$ -actinin and talin was less pronounced. No accumulation of actin-associated proteins was observed in uninfected (control) cervical epithelial cells. Magnification, X20.

**FIG. 9.** *Neisseria gonorrhoeae* co-localizes with CR3 *in vivo*. Cryosections of a clinical biopsy derived from a women with documented gonococcal cervicitis were immunolabeled with anti-CD18 (visible as a green fluorescence) and 2C3 (specific for gonococcal H.8 outer membrane protein, visible as a red fluorescence) antibodies. Co-localization of CR3 with gonococci occurs as a yellow fluorescence because of the combined signal of the two fluorophores. A) 63X oil B) 5X zoom image of the area designated by the white box in A. Co-localization is confirmed as a profile plot of the area designated by the red line where the individual fluorescence of each fluorophore is recorded and plotted, individually, by the viewing system. C) Areas of confirmed co-localization are observed where the peaks of the lines of the graph overlap.

**FIG. 10.** Bacterial products that are released with gonococcal infection.

**FIG. 11.** Proteomic analysis of gonococcal products released upon infection of primary cervical epithelia.

**FIG. 12** depicts a coomassie-stained polyacrylamide gel showing that the gonococcal products released from a cervical cell infection are not released with an



infection of male urethral cells. Supernatants were obtained from 90 minute and 3 hour infections of ecto- and endocervical cells.

**FIG. 13** depicts histograms from quantitative association and invasion assays that show that PLD-deficient gonococci are impaired in their adhere to and to  
5 invade primary cervical cells.

**FIG. 14** depicts photographs from confocal microscopy showing that PLD-deficient gonococci are impaired in their ability to elicit increased levels of CR3 surface expression on primary cervical cells. CR3 (CD18, CR3  $\beta$ -subunit) was immunolabeled with a TRITC-conjugated antibody and is visible as a red  
10 fluorescence; gonococci were immunolabeled with an antibody to the highly conserved outer membrane protein, H.8. Application of a FITC-conjugated secondary antibody allowed visualization of gonococci as a green fluorescence. Co-localization of CR3 with gonococci occurs as a yellow fluorescence because of the combined signal of the two fluorophores. Magnification 60x.

**FIG. 15** depicts histograms showing surface level expression of CR3 on primary cervical cells. Wild-type gonococci cells (1291-WT), but not PLD-deficient gonococci (1291 $\Delta$ PLD) elicit increased levels of CR3 surface expression. The antibody used was H5A4 ( $\alpha$ -I-domain) diluted 1/400.

**FIG. 16.** To determine if gonococcal PLD plays a role in the cytoskeletal  
20 rearrangements leading to membrane ruffling of the cervical epithelium, scanning electron microscopy (SEM) was performed. SEM analysis demonstrated that aberrant cytoskeletal rearrangements occur upon infection of cervical epithelia with PLD-mutant gonococci when compared to infection with wild-type gonococci. Endocytosis mediated by CR3 requires receptor clustering. The absence of bacterial  
25 clusters in electrographs taken of mutant gonococci at 3 hours post-infection (upper panel) may be reflective of the inability of these bacteria to elicit up-regulation of CR3 or of their inability to initiate signaling cascades required for CR3 clustering. Similarly, the absence of membrane ruffles (lower panel) in PLD infected cells suggests gonococcal PLD may be required to potentiate the cytoskeletal

rearrangements required to form membrane ruffles. These processes are restored when assays are performed with PLD-mutant gonococci in the presence of primed wild-type supernatants. No observable differences between mutant or wild-type gonococci were noted in the ability of gonococci to interact with each other or with cervical cells at earlier points of infection. Electrographs shown in the lower panel correspond to the respective boxed areas shown in the upper panel. Magnification: A) x 1k, B) x 1.1 k C) 800k D) x 9k, E) x 10k, and F) x 15k.

**FIG. 17** depicts Western blots of primary cervical cells infected with wild-type gonococci (1291-WT) (A, C) and PLD-deficient gonococci (1291 $\Delta$ PLD) (B, D). Cervical cell lysates were harvested at variable times post-infection (A and C: lane 1 (0 minutes), lane 2 (five minutes), lane 3 (10 minutes), lane 4 (15 minutes), lane 5 (30 minutes), lane 6 (45 minutes), lane 7 (60 minutes), lane 8 (90 minutes), lane 9 (2 hours), lane 10 (2.5 hours), lane 11 (3 hours), lane 12 (4 hours); B and D: lane 1 (4 hours), lane 2 (3 hours), lane 3 (2.5 hours), lane 4 (2 hours), lane 5 (90 minutes), lane 6 (60 minutes), lane 7 (45 minutes), lane 8 (30 minutes), lane 9 (15 minutes), lane 10 (10 minutes), lane 11 (five minutes), lane 12 (0 minutes)). Blots were probed with antibodies specific for phosphorylated tyrosine (A, B) or threonine target residues (C, D).

**FIG. 18** depicts multiplex RT-PCR for analysis of cytokine cDNA in primary human cervical cells. Lane 1 is DNA from uninfected primary cells (endo- or ectocervical (as noted)); lane 2 is primary cells infected with wild-type gonococcal cells (1291) and lane 3 is primary cells (endo- or ectocervical (as noted)) infected with PLD-deficient gonococcal cells (1291 PLD mutant).

**FIG. 19** depicts multiplex RT-PCR for cytokine cDNA analysis in secondary bronchial epithelial cells. Lane 1 is DNA from uninfected bronchial epithelial cells; lane 2 is bronchial epithelial cells infected with wild-type *Neisseria meningitidis* type B (NMB WT) and lane 3 is bronchial epithelial cells infected with PLD-deficient *Neisseria meningitidis* type B (NMB PLD mutant).

**FIG. 20** depicts histograms showing that tyrosine kinase activation partially rescues phenotypic PLD-deficiency observed with *N. gonorrhoeae* infection of primary cervical cells.

**FIG. 21** depicts histograms showing that protein kinase C activation rescues phenotypic PLD-deficiency observed with *N. gonorrhoeae* infection of primary cervical cells.

**FIG. 22** shows that gonococcal products released with cervical cell infection are not released with infection of male urethral cells. Analysis of infection supernatants demonstrated that gonococcal products are released upon infection of cervical epithelia. Similar results are observed upon analysis of supernatants obtained from 90 minute and 3 hour infections, from pex (A) and pen (B) cells, and from these same cells obtained from different tissue donors. An identical protein pattern is observed with *N. gonorrhoeae* strains 1291 (shown), FA1090, or MS11 indicating protein release is not strain-dependent. To determine if gonococcal proteins released upon cervical infection were specific to cervical cell invasion, these studies were repeated using male urethral epithelial cells. Autoradiography revealed that, while a minimal amount of protein products is released by 90 minutes post-infection, these proteins are not present by 3 hours of infection of uec (C). Collectively, these data suggest that a small basal level of gonococcal products are released constitutively, but, also that the continued release of gonococcal products was specific to gonococcal cervicitis. Western Blot analysis failed to reveal the presence of gonococcal LOS in culture supernatants, indicating the protein products identified were not present as the result of bacterial lysis (D). These data demonstrate the exquisite ability of the gonococcus to sense its extracellular environment and modify its pathogenicity accordingly. Lanes: C1) gonococci incubated in tissue culture dishes devoid of cervical cells, C2) uninfected cervical cells, C3) 90 minutes infection of uec, C4) 3 hours infection of uec, D1) 3 hours infection of pen cells, D2) 90 minutes infection of pen, D3) uninfected pen cells, and D4) *N. gonorrhoeae* LOS.

**FIG. 23.** RT-PCR analysis demonstrates that endogenous cervical cell PLD activity does not account for the observed increased in total PLD activity in gonococci infected cervical cell. Lanes: 1) uninfected pex or pen cells, 2) 3 hours infection of pex or pen cells with wild-type *N. gonorrhoeae* strain 1291, and 3) 3 hours infection of pex or pen cells with *N. gonorrhoeae* strain 1291ΔPLD.

### **Detailed Description of the Invention**

#### **Definitions**

As used herein, “disrupted *pld* gene” or “disrupted gene” refers to an insertion, substitution, or deletion either in the gene encoding phospholipase D or in the vicinity of the gene, *i.e.*, upstream (5') or downstream (3') of the gene, which results in the reduction of the biological activity or the loss of substantially all of the biological activity associated with the gene's product. For example, a disrupted *pld* gene would be unable to express a protein having substantial phospholipase D activity. By disrupting a neisserial *pld* gene, neisserial phospholipase D synthesis and/or function, *e.g.*, enzymatic activity related to PLD such as the catalysis of phospholipase D-related hydrolysis and/or phosphatidyl-transferase reactions, is reduced, *e.g.*, inhibited, as compared to wild-type biological activity. Methods for measuring PLD activity are known in the art. The synthesis and/or function of a *pld* gene can be inhibited by any one of a number of methods known to the art, for example, by administration of chemical inhibitors of protein synthesis, by site-directed mutagenesis, by antisense methodology or using siRNA techniques. For example, PLD synthesis and/or function can be inhibited by the “disruption” of a gene encoding a neisserial PLD, *e.g.*, by insertion, substitution and/or deletion, in the *pld* gene or in a gene in the vicinity, *i.e.*, either upstream (5') or downstream (3') of the *pld* gene, which results in the reduction of the biological activity or the loss of substantially all of the biological activity associated with the gene's product.

As used herein, the term “neisserial PLD” includes homologs, variants or biologically active or inactive fragments of PLD from any *Neisserial spp.*, *e.g.*, *N.*

*gonorrhoeae* or PLD from *N. meningitidis*. A “variant” of the polypeptide is a neisserial protein that is not completely identical to a native neisserial protein. A variant neisserial protein can be obtained by altering the amino acid sequence by insertion, deletion or substitution of one or more amino acid. The amino acid

5 sequence of the protein is modified, for example by substitution, to create a polypeptide having substantially the same or improved qualities as compared to the native (*i.e.*, wild type) polypeptide. The substitution may be a conserved substitution. A “conserved substitution” is a substitution of an amino acid with another amino acid having a similar side chain. A conserved substitution would be

10 a substitution with an amino acid that makes the smallest change possible in the charge of the amino acid or size of the side chain of the amino acid (alternatively, in the size, charge or kind of chemical group within the side chain) such that the overall peptide retains its spacial conformation but has altered biological activity. For example, common conserved changes might be Asp to Glu, Asn or Gln; His to

15 Lys, Arg or Phe; Asn to Gln, Asp or Glu and Ser to Cys, Thr or Gly. Alanine is commonly used to substitute for other amino acids. The 20 essential amino acids can be grouped as follows: alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan and methionine having nonpolar side chains; glycine, serine, threonine, cystine, tyrosine, asparagine and glutamine having uncharged

20 polar side chains; aspartate and glutamate having acidic side chains; and lysine, arginine, and histidine having basic side chains. Stryer, L. *Biochemistry* (2d edition) W. H. Freeman and Co. San Francisco (1981), p. 14-15; Lehninger, A. *Biochemistry* (2d ed., 1975), p. 73-75.

It is known that variant polypeptides can be obtained based on substituting

25 certain amino acids for other amino acids in the polypeptide structure in order to modify or improve biological activity. For example, through substitution of alternative amino acids, small conformational changes may be conferred upon a polypeptide that result in increased bioactivity. Alternatively, amino acid substitutions in certain polypeptides may be used to provide residues that may then

be linked to other molecules to provide peptide-molecule conjugates that retain sufficient properties of the starting polypeptide to be useful for other purposes.

One can use the hydropathic index of amino acids in conferring interactive biological function on a polypeptide, wherein it is found that certain amino acids  
5 may be substituted for other amino acids having similar hydropathic indices and still retain a similar biological activity. Alternatively, substitution of like amino acids may be made on the basis of hydrophilicity, particularly where the biological function desired in the polypeptide to be generated is intended for use in immunological embodiments. The greatest local average hydrophilicity of a protein,  
10 as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity. U.S. Patent 4,554,101. Accordingly, it is noted that substitutions can be made based on the hydrophilicity assigned to each amino acid. In using either the hydrophilicity index or hydropathic index, which assigns values to each amino acid, substitutions may be conducted, for example, where these values are  $\pm 2$ ,  
15  $\pm 1$  or  $\pm 0.5$ .

The variant neisserial protein comprises at least seven amino acid residues, preferably about 20 to about 2000 residues, and more preferably about 50 to about 1000 residues, and even more preferably about 80 to about 200 residues, wherein the variant neisserial protein has at least 50%, preferably at least about 80%, and more  
20 preferably at least about 90% but less than 100%, contiguous amino acid sequence homology or identity to the amino acid sequence of a corresponding native neisserial protein.

The amino acid sequence of the variant neisserial protein corresponds essentially to the native neisserial protein amino acid sequence. As used herein  
25 “correspond essentially to” refers to a polypeptide sequence that will elicit a protective immunological response substantially the same as the response generated by native neisserial protein. Such a response may be at least 60% of the level generated by native neisserial protein, and may even be at least 80% of the level generated by native neisserial protein. An immunological response to a composition

or vaccine is the development in the host of a cellular and/or antibody-mediated immune response to the polypeptide or vaccine of interest. Usually, such a response consists of the subject producing antibodies, B cell, helper T cells, suppressor T cells, and/or cytotoxic T cells directed specifically to an antigen or antigens included  
5 in the composition or vaccine of interest.

A variant of the invention may include amino acid residues not present in the corresponding native neisserial protein, or may include deletions relative to the corresponding native neisserial protein. A variant may also be a truncated “fragment” as compared to the corresponding native neisserial protein, *i.e.*, only a  
10 portion of a full-length protein. Neisserial protein variants also include peptides having at least one D-amino acid.

The neisserial protein of the present invention may be expressed from an isolated nucleic acid (DNA or RNA) sequence encoding the neisserial protein. Amino acid changes from the native to the variant neisserial protein may be  
15 achieved by changing the codons of the corresponding nucleic acid sequence. “Recombinant” is defined as a peptide or nucleic acid produced by the processes of genetic engineering. It should be noted that it is well-known in the art that, due to the redundancy in the genetic code, individual nucleotides can be readily exchanged in a codon, and still result in an identical amino acid sequence. The terms “protein,”  
20 “peptide” and “polypeptide” are used interchangeably herein.

The neisserial protein as described above may be operably linked to an amino acid sequence for a therapeutic agent. An amino acid or nucleic acid is “operably linked” when it is placed into a functional relationship with another amino acid or nucleic acid sequence. For example, DNA a pre-sequence or secretory  
25 leader is operably linked to DNA for a polypeptide if it is expressed as a pre-protein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, “operably linked” means that the amino

acid or nucleic acid sequences being linked are contiguous, and, in the case of a secretory leader in DNA, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or  
5 linkers are used in accordance with conventional practice.

The term “nucleic acid” refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form, composed of monomers (nucleotides) containing a sugar, phosphate and a base which is either a purine or pyrimidine. Unless specifically limited, the term encompasses nucleic  
10 acids containing known analogs of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences as well as the  
15 sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucl. Acids Res., 19:508 (1991); Ohtsuka et al., JBC, 260:2605 (1985); Rossolini et al., Mol. Cell. Probes, 8:91 (1994)). A “nucleic acid fragment”  
20 is a fraction of a given nucleic acid molecule. Deoxyribonucleic acid (DNA) in the majority of organisms is the genetic material while ribonucleic acid (RNA) is involved in the transfer of information contained within DNA into proteins. The term “nucleotide sequence” refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered  
25 nucleotide bases capable of incorporation into DNA or RNA polymers. The terms “nucleic acid”, “nucleic acid molecule”, “nucleic acid fragment”, “nucleic acid sequence or segment”, or “polynucleotide” may also be used interchangeably with gene, cDNA, DNA and RNA encoded by a gene.



The invention encompasses isolated or substantially purified nucleic acid or protein compositions. In the context of the present invention, an "isolated" or "purified" DNA molecule or an "isolated" or "purified" polypeptide is a DNA molecule or polypeptide that exists apart from its native environment and is therefore not a product of nature. An isolated DNA molecule or polypeptide may exist in a purified form or may exist in a non-native environment such as, for example, a transgenic host cell. For example, an "isolated" or "purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. In one embodiment, an "isolated" nucleic acid is free of sequences that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A protein that is substantially free of cellular material includes preparations of protein or polypeptide having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating protein. When the protein of the invention, or biologically active portion thereof, is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein-of-interest chemicals. Fragments and variants of the disclosed nucleotide sequences and proteins or partial-length proteins encoded thereby are also encompassed by the present invention. By "fragment" or "portion" is meant a full length or less than full length of the nucleotide sequence encoding, or the amino acid sequence of, a polypeptide or protein.

The term "gene" is used broadly to refer to any segment of nucleic acid associated with a biological function. Thus, genes include coding sequences and/or

the regulatory sequences required for their expression. For example, gene refers to a nucleic acid fragment that expresses mRNA, functional RNA, or specific protein, including regulatory sequences. Genes also include nonexpressed DNA segments that, for example, form recognition sequences for other proteins. Genes can be  
5 obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters.

"Naturally occurring" is used to describe an object that can be found in nature as distinct from being artificially produced. For example, a protein or  
10 nucleotide sequence present in an organism (including a virus), which can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory, is naturally occurring.

The term "chimeric" refers to any gene or DNA that contains 1) DNA sequences, including regulatory and coding sequences, that are not found together in  
15 nature, or 2) sequences encoding parts of proteins not naturally adjoined, or 3) parts of promoters that are not naturally adjoined. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or comprise regulatory sequences and coding sequences derived from the same source, but arranged in a manner different from that found in nature.

20 A "transgene" refers to a gene that has been introduced into the genome by transformation and is stably maintained. Transgenes may include, for example, DNA that is either heterologous or homologous to the DNA of a particular cell to be transformed. Additionally, transgenes may comprise native genes inserted into a non-native organism, or chimeric genes. The term "endogenous gene" refers to a  
25 native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism but that is introduced by gene transfer.

The terms "protein," "peptide" and "polypeptide" are used interchangeably herein.

A "variant" of a molecule is a sequence that is substantially similar to the sequence of the native molecule. For nucleotide sequences, variants include those sequences that, because of the degeneracy of the genetic code, encode the identical amino acid sequence of the native protein. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis which encode the native protein, as well as those that encode a polypeptide having amino acid substitutions.

"Conservatively modified variations" of a particular nucleic acid sequence refers to those nucleic acid sequences that encode identical or essentially identical amino acid sequences, or where the nucleic acid sequence does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance the codons CGT, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded protein. Such nucleic acid variations are "silent variations" which are one species of "conservatively modified variations." Every nucleic acid sequence described herein which encodes a polypeptide also describes every possible silent variation, except where otherwise noted. One of skill will recognize that each codon in a nucleic acid (except ATG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

"Recombinant DNA molecule" is a combination of DNA sequences that are joined together using recombinant DNA technology and procedures used to join

together DNA sequences as described, for example, in Sambrook and Russell (2001).

The terms "heterologous DNA sequence," "exogenous DNA segment" or "heterologous nucleic acid," each refer to a sequence that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell but has been modified. The terms also include non-naturally occurring multiple copies of a naturally occurring DNA sequence. Thus, the terms refer to a DNA segment that is foreign or heterologous to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides.

A "homologous" DNA sequence is a DNA sequence that is naturally associated with a host cell into which it is introduced.

"Wild-type" refers to the native gene or organism as found in nature.

"Genome" refers to the complete genetic material of an organism.

A "vector" is defined to include, *inter alia*, any plasmid, cosmid, phage or binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g., autonomous replicating plasmid with an origin of replication).

"Cloning vectors" typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance, hygromycin resistance or ampicillin resistance.

"Expression cassette" as used herein means a DNA sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. It also typically comprises sequences  
5 required for proper translation of the nucleotide sequence. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or a nontranslated RNA, in the sense or antisense direction. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components is  
10 heterologous with respect to at least one of its other components. The expression cassette may also be one which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter which initiates transcription only  
15 when the host cell is exposed to some particular external stimulus. In the case of a multicellular organism, the promoter can also be specific to a particular tissue or organ or stage of development.

Such expression cassettes will comprise the transcriptional initiation region of the invention linked to a nucleotide sequence of interest. Such an expression  
20 cassette is provided with a plurality of restriction sites for insertion of the gene of interest to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

"Coding sequence" refers to a DNA or RNA sequence that codes for a specific amino acid sequence and excludes the non-coding sequences. It may  
25 constitute an "uninterrupted coding sequence", i.e., lacking an intron, such as in a cDNA or it may include one or more introns bounded by appropriate splice junctions. An "intron" is a sequence of RNA which is contained in the primary transcript but which is removed through cleavage and re-ligation of the RNA within the cell to create the mature mRNA that can be translated into a protein.

The terms "open reading frame" and "ORF" refer to the amino acid sequence encoded between translation initiation and termination codons of a coding sequence. The terms "initiation codon" and "termination codon" refer to a unit of three adjacent nucleotides ('codon') in a coding sequence that specifies initiation and chain termination, respectively, of protein synthesis (mRNA translation).

A "functional RNA" refers to an antisense RNA, ribozyme, or other RNA that is not translated.

The term "RNA transcript" refers to the product resulting from RNA polymerase catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA" (mRNA) refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a single- or a double-stranded DNA that is complementary to and derived from mRNA.

"Regulatory sequences" and "suitable regulatory sequences" each refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences include enhancers, promoters, translation leader sequences, introns, and polyadenylation signal sequences. They include natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural sequences. As is noted above, the term "suitable regulatory sequences" is not limited to promoters. However, some suitable regulatory sequences useful in the present invention will include, but are not limited to constitutive promoters, tissue-specific promoters, development-specific promoters, inducible promoters and viral promoters.

"5' non-coding sequence" refers to a nucleotide sequence located 5' (upstream) to the coding sequence. It is present in the fully processed mRNA

upstream of the initiation codon and may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency (Turner et al., Mol. Biotech., 3:225 (1995).

5 "3' non-coding sequence" refers to nucleotide sequences located 3' (downstream) to a coding sequence and include polyadenylation signal sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

10 The term "translation leader sequence" refers to that DNA sequence portion of a gene between the promoter and coding sequence that is transcribed into RNA and is present in the fully processed mRNA upstream (5') of the translation start codon. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency.

15 The term "mature" protein refers to a post-translationally processed polypeptide without its signal peptide. "Precursor" protein refers to the primary product of translation of an mRNA. "Signal peptide" refers to the amino terminal extension of a polypeptide, which is translated in conjunction with the polypeptide forming a precursor peptide and which is required for its entrance into the secretory  
20 pathway. The term "signal sequence" refers to a nucleotide sequence that encodes the signal peptide.

"Promoter" refers to a nucleotide sequence, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper  
25 transcription. "Promoter" includes a minimal promoter that is a short DNA sequence comprised of a TATA- box and other sequences that serve to specify the site of transcription initiation, to which regulatory elements are added for control of expression. "Promoter" also refers to a nucleotide sequence that includes a minimal promoter plus regulatory elements that is capable of controlling the expression of a

coding sequence or functional RNA. This type of promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a DNA sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue specificity of a promoter. It is capable of operating in both orientations (normal or flipped), and is capable of functioning even when moved either upstream or downstream from the promoter. Both enhancers and other upstream promoter elements bind sequence-specific DNA-binding proteins that mediate their effects. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even be comprised of synthetic DNA segments. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions.

15       The "initiation site" is the position surrounding the first nucleotide that is part of the transcribed sequence, which is also defined as position +1. With respect to this site all other sequences of the gene and its controlling regions are numbered. Downstream sequences (i.e. further protein encoding sequences in the 3' direction) are denominated positive, while upstream sequences (mostly of the controlling regions in the 5' direction) are denominated negative.

20       Promoter elements, particularly a TATA element, that are inactive or that have greatly reduced promoter activity in the absence of upstream activation are referred to as "minimal or core promoters." In the presence of a suitable transcription factor, the minimal promoter functions to permit transcription. A "minimal or core promoter" thus consists only of all basal elements needed for transcription initiation, e.g., a TATA box and/or an initiator.

25       "Constitutive expression" refers to expression using a constitutive or regulated promoter. "Conditional" and "regulated expression" refer to expression controlled by a regulated promoter.



"Operably-linked" refers to the association of nucleic acid sequences on single nucleic acid fragment so that the function of one is affected by the other. For example, a regulatory DNA sequence is said to be "operably linked to" or "associated with" a DNA sequence that codes for an RNA or a polypeptide if the

5 two sequences are situated such that the regulatory DNA sequence affects expression of the coding DNA sequence (i.e., that the coding sequence or functional RNA is under the transcriptional control of the promoter). Coding sequences can be operably-linked to regulatory sequences in sense or antisense orientation.

"Expression" refers to the transcription and/or translation of an endogenous

10 gene or a transgene in cells. For example, in the case of antisense constructs, expression may refer to the transcription of the antisense DNA only. In addition, expression refers to the transcription and stable accumulation of sense (mRNA) or functional RNA. Expression may also refer to the production of protein.

"Transcription stop fragment" refers to nucleotide sequences that contain one

15 or more regulatory signals, such as polyadenylation signal sequences, capable of terminating transcription. Examples include the 3' non-regulatory regions of genes encoding nopaline synthase and the small subunit of ribulose biphosphate carboxylase.

"Translation stop fragment" refers to nucleotide sequences that contain one

20 or more regulatory signals, such as one or more termination codons in all three frames, capable of terminating translation. Insertion of a translation stop fragment adjacent to or near the initiation codon at the 5' end of the coding sequence will result in no translation or improper translation. Excision of the translation stop fragment by site-specific recombination will leave a site-specific sequence in the

25 coding sequence that does not interfere with proper translation using the initiation codon.

The terms "*cis*-acting sequence" and "*cis*-acting element" refer to DNA or RNA sequences whose functions require them to be on the same molecule.

The terms "*trans*-acting sequence" and "*trans*-acting element" refer to DNA or RNA sequences whose function does not require them to be on the same molecule.

"Chromosomally-integrated" refers to the integration of a foreign gene or DNA construct into the host DNA by covalent bonds. Where genes are not "chromosomally integrated" they may be "transiently expressed." Transient expression of a gene refers to the expression of a gene that is not integrated into the host chromosome but functions independently, either as part of an autonomously replicating plasmid or expression cassette, for example, or as part of another biological system such as a virus.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be

accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller, CABIOS, 4:11 (1988); the local homology algorithm of Smith et al., Adv. Appl. Math., 2:482 (1981); the homology alignment algorithm of Needleman and Wunsch, JMB, 48:443 (1970); the search-for-similarity-method of Pearson and Lipman, Proc. Natl. Acad. Sci. USA, 85:2444 (1988); the algorithm of Karlin and Altschul, Proc. Natl. Acad. Sci. USA, 87:2264 (1990), modified as in Karlin and Altschul, Proc. Natl. Acad. Sci. USA, 90:5873 (1993).

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins et al., Gene, 73:237 (1988); Higgins et al., CABIOS, 5:151 (1989); Corpet et al., Nucl. Acids Res., 16:10881 (1988); Huang et al., CABIOS, 8:155 (1992); and Pearson et al., Meth. Mol. Biol., 24:307 (1994). The ALIGN program is based on the algorithm of Myers and Miller, *supra*. The BLAST programs of Altschul et al., JMB, 215:403 (1990); Nucl. Acids Res., 25:3389 (1990), are based on the algorithm of Karlin and Altschul *supra*.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches

to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached.

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al., Nucleic Acids Res. 25:3389 (1997). Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul et al., *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g. BLASTN for nucleotide sequences, BLASTX for proteins) can be used. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an

expectation (E) of 10, and the BLOSUM62 scoring matrix. See <http://www.ncbi.nlm.nih.gov>. Alignment may also be performed manually by inspection.

For purposes of the present invention, comparison of nucleotide sequences  
5 for determination of percent sequence identity to the promoter sequences disclosed  
herein can be made using the BlastN program (version 1.4.7 or later) with its default  
parameters or any equivalent program. By "equivalent program" is intended any  
sequence comparison program that, for any two sequences in question, generates an  
alignment having identical nucleotide or amino acid residue matches and an  
10 identical percent sequence identity when compared to the corresponding alignment  
generated by the BlastN program.

(c) As used herein, "sequence identity" or "identity" in the context of two  
nucleic acid or polypeptide sequences makes reference to a specified percentage of  
residues in the two sequences that are the same when aligned for maximum  
15 correspondence over a specified comparison window, as measured by sequence  
comparison algorithms or by visual inspection. When percentage of sequence  
identity is used in reference to proteins it is recognized that residue positions which  
are not identical often differ by conservative amino acid substitutions, where amino  
acid residues are substituted for other amino acid residues with similar chemical  
20 properties (e.g., charge or hydrophobicity) and therefore do not change the  
functional properties of the molecule. When sequences differ in conservative  
substitutions, the percent sequence identity may be adjusted upwards to correct for  
the conservative nature of the substitution. Sequences that differ by such  
conservative substitutions are said to have "sequence similarity" or "similarity."  
25 Means for making this adjustment are well known to those of skill in the art.  
Typically this involves scoring a conservative substitution as a partial rather than a  
full mismatch, thereby increasing the percentage sequence identity. Thus, for  
example, where an identical amino acid is given a score of 1 and a non-conservative  
substitution is given a score of zero, a conservative substitution is given a score

between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, or 79%, preferably at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more preferably at least 90%, 91%, 92%, 93%, or 94%, and most preferably at least 95%, 96%, 97%, 98%, or 99% sequence identity, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 70%, more preferably at least 80%, 90%, and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions (see below). Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength and pH.

However, stringent conditions encompass temperatures in the range of about 1 °C to about 20 °C, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

- 5  
10 (e)(ii) The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, or 79%, preferably 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more preferably at least 90%, 91%, 92%, 93%, or 94%, or even more preferably, 95%, 96%, 97%, 98% or 99%, sequence identity to the reference  
15 sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443 (1970). An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially  
20 identical to a second peptide, for example, where the two peptides differ only by a conservative substitution.

- For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer,  
25 subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

As noted above, another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. The phrase "hybridizing specifically to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA. "Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target nucleic acid sequence.

"Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern hybridizations are sequence dependent, and are different under different environmental parameters. Longer sequences hybridize specifically at higher temperatures. The thermal melting point ( $T_m$ ) is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the  $T_m$  can be approximated from the equation of Meinkoth and Wahl, Anal. Biochem., 138:267 (1984);  $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$ ; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs.  $T_m$  is reduced by about  $1^\circ\text{C}$  for each 1% of mismatching; thus,  $T_m$ , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with >90% identity are sought, the  $T_m$  can be decreased  $10^\circ\text{C}$ . Generally, stringent conditions are selected to be about  $5^\circ\text{C}$  lower than the  $T_m$  for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent



conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the  $T_m$ ; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the  $T_m$ ; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the  $T_m$ . Using the equation, hybridization and wash compositions, and desired temperature, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a temperature of less than 45°C (aqueous solution) or 32°C (formamide solution), the SSC concentration can be increased so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology Hybridization with Nucleic Acid Probes, part I chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays" Elsevier, New York (1993). Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the  $T_m$  for the specific sequence at a defined ionic strength and pH.

An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2X SSC wash at 65°C for 15 minutes (see, Sambrook and Russell, *infra*, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1X SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4-6X SSC at 40°C for 15 minutes. For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.5 M, for example, about 0.01 to 1.0 M, Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30°C and at least about 60°C for long probes (e.g., >50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. In general,

a signal to noise ratio of 2X (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the proteins that they encode are substantially identical.

- 5 This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

- Very stringent conditions are selected to be equal to the  $T_m$  for a particular probe. An example of stringent conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a
- 10 Southern or Northern blot is 50% formamide, e.g., hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C.
- 15 Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C.

- By "variant" polypeptide is intended a polypeptide derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to
- 20 the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Such variants may results form, for example, genetic polymorphism or from human manipulation. Methods for such manipulations are generally known in the art.

- 25 Thus, the polypeptides of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the polypeptides can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the

art. See, for example, Kunkel, Proc. Natl. Acad. Sci. USA, 82:488 (1985); Kunkel et al., Meth. Enzymol., 154:367 (1987); U. S. Patent No. 4,873,192; Walker and Gaastra, Techniques in Mol. Biol. (MacMillan Publishing Co. (1983), and the references cited therein. Guidance as to appropriate amino acid substitutions that do  
5 not affect biological activity of the protein of interest may be found in the model of Dayhoff et al., Atlas of Protein Sequence and Structure (Natl. Biomed. Res. Found. 1978). In one embodiment of the invention, conservative substitutions, such as exchanging one amino acid with another having similar properties, are made.

Thus, the genes and nucleotide sequences of the invention include both the  
10 naturally occurring sequences as well as mutant forms. Likewise, the polypeptides of the invention encompass both naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the desired activity. The deletions, insertions, and substitutions of the polypeptide sequence encompassed herein are not expected to produce radical changes in the  
15 characteristics of the polypeptide. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays.

Individual substitutions deletions or additions that alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more  
20 typically less than 1%) in an encoded sequence are "conservatively modified variations," where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following five groups each contain amino acids that are conservative substitutions for one another:  
25 Aliphatic: Glycine (G), Alanine (A), Valine (V), Leucine (L), Isoleucine (I);  
Aromatic: Phenylalanine (F), Tyrosine (Y), Tryptophan (W); Sulfur-containing: Methionine (M), Cysteine (C); Basic: Arginine (R), Lysine (K), Histidine (H);  
Acidic: Aspartic acid (D), Glutamic acid (E), Asparagine (N), Glutamine (Q). In addition, individual substitutions, deletions or additions which alter, add or delete a

single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations."

The term "transformation" refers to the transfer of a nucleic acid fragment into the genome of a host cell, resulting in genetically stable inheritance. Host cells  
5 containing the transformed nucleic acid fragments are referred to as "transgenic" cells, and organisms comprising transgenic cells are referred to as "transgenic organisms".

"Transformed," "transgenic," and "recombinant" refer to a host cell or organism into which a heterologous nucleic acid molecule has been introduced. The  
10 nucleic acid molecule can be stably integrated into the genome generally known in the art and are disclosed in Sambrook et al., Molecular Cloning: A Laboratory Manual (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York) (1989). See also Innis et al., PCR Protocols, Academic Press (1995); and Gelfand, PCR Strategies, Academic Press (1995); and Innis and Gelfand, PCR Methods  
15 Manual, Academic Press (1999). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially mismatched primers, and the like. For example, "transformed," "transformant," and "transgenic" cells have been through the transformation process and contain a  
20 foreign gene integrated into their chromosome. The term "untransformed" refers to normal cells that have not been through the transformation process.

A "transgenic" organism is an organism having one or more cells that contain an expression vector.

By "portion" or "fragment", as it relates to a nucleic acid molecule, sequence  
25 or segment of the invention, when it is linked to other sequences for expression, is meant a sequence having at least 80 nucleotides, more preferably at least 150 nucleotides, and still more preferably at least 400 nucleotides. If not employed for expressing, a "portion" or "fragment" means at least 9, preferably 12, more preferably 15, even more preferably at least 20, consecutive nucleotides, e.g., probes

and primers (oligonucleotides), corresponding to the nucleotide sequence of the nucleic acid molecules of the invention.

As used herein, the term “therapeutic agent” refers to any agent or material that has a beneficial effect on the mammalian recipient. Thus, “therapeutic agent”  
5 embraces both therapeutic and prophylactic molecules having nucleic acid or protein components.

The term “antibody” includes intact molecules of polyclonal or monoclonal antibodies, as well as fragments thereof, such as Fab and F(ab')<sub>2</sub>. For example, monoclonal antibodies are made from antigen containing fragments of a protein by  
10 methods well known to those skilled in the art (Kohler et al., Nature, 256, 495 (1975)).

By “immunize” is meant to stimulate an immune response (humoral and/or cellular), *e.g.*, such that may render immune a vaccine recipient. “Immunization” refers to the production of antibodies directed against an infecting agent and/or its  
15 toxic product. It may also initiate a cellular response. For example, a vaccine of the invention may be used to immunize a mammal, such as a human, against current or subsequent infection caused by one or more *Neisseria spp.* A vaccine of the invention is effective for eliciting antibodies that are immunoreactive with a *Neisseria spp.* that expresses one or more phospholipase D protein(s).

20

#### **Direct Association of CR3 with Pathogenic *Neisseria***

Phagocytosis that is mediated by complement receptor type 3 (CR3) occurs independently of a proinflammatory response in immune cells (Caron *et al.* 1998). CR3 exists as an integrin heterodimer composed of an alpha ( $\alpha_M$  or CD11b) and a  
25 beta ( $\beta_2$  or CD18) subunit. The distribution of CR3 is thought to be limited to professional phagocytes; however, Hussain *et al.* (1995) demonstrated the expression of CR3 in rectal epithelia. Additionally, Hussain *et al.* (1995) were able to detect the presence of CD11b in a small subpopulation of cervicovaginal epithelia, although they were unable to detect the presence of CD18.

Up-regulation of CD11b in neutrophils has been documented in response to *Neisseria meningitidis* infection (Kraghsbjerg *et al.* 2000). The direct association of CR3 with pathogenic *Neisseria*, however, has not been demonstrated. The present inventors herein describe the occurrence of CR3 expression in primary human cervical epithelial cells and its co-localization with *N. gonorrhoeae* upon infection of these primary epithelial cells. They also describe the distribution of CR3 in immortalized tissue culture cell lines and within tissue biopsies derived from the male and female urogenital tracts. Monoclonal antibodies directed against CR3 inhibit gonococcal invasion of primary cervical cells and of CR3-transfected CHO cells suggesting that CR3 serves as a receptor for *N. gonorrhoeae* during infection. In addition, these studies help to explain why the inflammatory response initiated by gonococcal infection of the lower female genital tract differs from that observed with gonococcal infection of the male urogenital tract.

The distribution of CR3 in tissue biopsies derived from defined sites within the human male and female genital tracts and in primary, immortalized, and malignant epithelial cells derived from these sites is described in Example 2 below. Laser scanning confocal microscopy (LSCM) demonstrated CR3 was not present in tissues and cells derived from the male urogenital tract and from tissue derived from the female urethra; however, CR3 was present on tissues and cells derived from the female genital tract. CR3 expression was greatest within the ectocervix tissue. Surface levels of CR3 appeared to decrease progressively from the ectocervix to the upper female genital tract in these tissues. A low level of CR3-associated immunofluorescence was observed in fallopian tube tissue. Consistent with results obtained with LSCM analysis of tissue biopsies, primary endo- and ectocervical cells possessed both CR3 subunits, and CR3 expression appeared to be greater on primary ectocervical cells in comparison to primary endocervical cells.

In contrast to results obtained with analysis of tissue biopsies and primary cervical cells, CR3 expression was negligible on immortalized and malignant cell lines (*i.e.*, HCK, End1, ME180, Hec1B). Infection studies using *N. gonorrhoeae*

strains 1291, 1291-green, MS11-green, or FA1090-green did not significantly influence the level of CR3 surface expression on these immortalized or malignant cell lines. However, *N. gonorrhoeae* did appear to induce up-regulation of CR3 surface expression on primary endo- and ectocervical cells. Gonococci were  
5 observed to co-localize with CR3 on primary cervical cells, and co-localization became increasingly prominent with extended infection. Immunoprecipitation studies confirmed the presence of CD11b and CD18 in primary cervical cells and CR3 co-localization with the gonococcus. Gonococci bound CR3-transfected K562 and CHO cells, and binding could be inhibited by the presence of anti-CD11b or -  
10 CD18 antibodies (FIG. 1). Similarly, invasion of primary cervical cells and CHO-CR3 cells could be inhibited by the addition of anti-CR3 antibodies to gentamicin-survival assays (FIGS 1 and 2). Gonococcal invasion of primary endo- and ectocervical cells was also inhibited by the addition of *Clostridium* C3 neurotoxin to invasion assays (FIG. 3), which is consistent with CR3-mediated phagocytosis  
15 (Caron *et al.* 1998).

Extensive membrane ruffling could be induced to occur in the absence of gonococci in primary endo- and ectocervical cells and in CHO-CR3 cells by the addition of anti-CD11b or -CD18 antibodies to infection assays. This suggests that engagement of CR3 elicits membrane ruffling, which occurs in response to *N.*  
20 *gonorrhoeae* infection of the cervical epithelium.

The role of complement (C') in innate immunity is multifactorial; however, C' predominately serves to eliminate foreign antigens and to regulate the inflammatory response directed towards these exogenous particles. C' protein C3 of the C' alternative pathway (AP) plays a paramount role in AP C' regulation in that it  
25 serves to amplify the complement-mediated response by a positive feedback regulatory loop, which converts a relatively inefficient response to a highly efficient defense mechanism. Activation of the AP occurs constitutively at a low rate, which is tightly regulated by C' regulatory proteins, *e.g.*, factors H (fH) and I (fI). Deposition of C3 on an exogenous surface (*e.g.* a bacterium) results in spontaneous

C3 hydrolysis to produce C3b. C3b can bind factor B (fB) to generate C3 convertase activity leading to the formation of the membrane attack complex. Alternatively, C3b can bind fH leading to C' inactivation via cleavage of C3b by fI to produce iC3b, a ligand for CR3.

5 CR3 distribution has generally been considered to be limited to immune cells (e.g., monocytes, neutrophils, and macrophages); however, CR3 has also been found on renal glomerular (Sandilands *et al.* 1985) and rectal (Hussain *et al.* 1995) epithelial cells. By *in situ* hybridization Hussain *et al.* (1995) detected CD11b in a sub-population of endocervical tissue specimens, but they were unable to detect  
10 CD18. The inability to detect CD18 was attributed to a level of CR3 expression that was below the sensitivity of the antibody and detection method used (Hussain *et al.* 1995). LSCM of surgical biopsies and of primary endo- and ectocervical cell monolayers (using two, well defined, antibodies to each CR3 subunit) demonstrated CR3 within the ectocervical, endocervical, endometrial, and fallopian tube epithelia;  
15 however, CR3 expression appeared to progressively decrease from the ectocervix to the fallopian tubes. Although CR3 is structurally and functionally related to the very late antigen (VLA) sub-family of integrins, which are present within the female genital tract (Sülz *et al.* 1998), these two distinct groups of proteins are not immunologically cross-reactive (Hynes, R. O. 1987). Additionally, isotype control  
20 antibodies failed to label primary cell monolayers or tissue cryosections.

The present inventors' immunohistochemical data provide evidence for the presence of CR3 within the female genital tract. Furthermore, immunoprecipitation of primary cervical cell lysates confirmed the presence of CR3 within the endo- and ectocervix by the presence of the appropriate 95 kDa (CD18) and 170 kDa (CD11b)  
25 bands with subsequent western blotting. These data suggest that the distribution of CR3 should now be extended to include the endo- and ectocervix and, possibly, the epithelia of the endometrium and fallopian tubes. The female reproductive tract and seminal fluid have been hypothesized to exhibit anomalous C' regulatory characteristics that exist to ensure successful reproduction by hindering an amplified



immune response to seminal plasma (Vanderpuye *et al.* 1992). Seminal plasma has been demonstrated to contain unidentified C1 and C3 C' component inhibitors, trace amounts of fH and fI, and a soluble form of the C3 regulatory protein, CD46 (Hussain *et al.* 1995), but fB has not been detected (Vanderpuye *et al.* 1992).

5           Full AP complement activity has been reported in cervical mucous (Price *et al.* 1979; Vanderpuye *et al.* 1992); however, C4 of the complement classical pathway (CP) was only detected in a small sub-population of luteal-phase cervical secretions (Vanderpuye *et al.* 1992). Additionally, AP, but not CP, components are produced by the vaginal epithelium (Price *et al.* 1979), and there are some data to  
10 suggest that C' components are synthesized by the endometrium (Vanderpuye *et al.* 1992). Collectively these data suggest that CR3 present within the female genital tract would function to eliminate exogenous antigens (with the absence of neutrophil influx), following C' inactivation of these antigens in seminal fluid or cervical mucous.

15           In contrast to the results obtained with female genital tissue and primary endo- and ectocervical cells, the presence of CR3 was not detected in vas deferens or male and female urethral tissue. The absence of CR3 in these tissues may be the result of divergent embryonic development that occurs after differentiation of the nephrogenic mesoderm. CR3 belongs to a large family of cell adhesion molecules  
20 that exhibit broad ligand specificity, and, in this respect, differential expression of integrin receptors has been implicated to play a role in morphogenesis and differentiation.

*Drosophila* spp. differentially express surface antigens, which structurally resemble human integrins, during the course of imaginal disc formation (Hynes, R.  
25 O. 1987). These cell surface molecules are hypothesized to influence embryonic development through differential cell adhesion (Hynes, R. O. 1987). In terms of evolutionary development, it is generally accepted that the female urogenital systems of apes and humans are more evolved than their male counterparts. In humans, the nephrogenic mesoderm differentiates to form the mesonephros and the

metanephros. The metanephros gives rise to the renal glomerulus while the mesonephros regresses. Remnants of the mesonephric tubules exist in males as the vas deferens and in females as blind tubules in the ovarian dorsal mesentery.

Muellerian ducts differentiate in females to form that portion of the female genital tract ranging from the fallopian tubes to the cephalic vagina. A complete division of the cloaca gives rise to the rectum and a urogenital sinus in both males and females. In males the muellerian ducts regress, and the urogenital sinus receives the mesonephric ducts, after which the rectum elongates and differentiation occurs. In females an additional portioning event of the urogenital sinus occurs to form the terminal vagina, the rectum, and the urethra. Since CR3 has been demonstrated on renal glomerular epithelium, rectal epithelium, and (considering the data of Hussain *et al.* (1995) and herein) the cervical epithelium, it is possible, although speculative, that the presence of CR3 in these tissues may correlate with a higher degree of embryonic development or cellular differentiation.

The absence of CR3 on the immortalized (End1, HCK) and the malignant (ME180, Hec1B) cell lines used in these studies may be reflective of the functional properties of integrins in general or CR3 specifically. Tumor cells are frequently altered in their integrin expression patterns (Jones *et al.* 1999) as well as the expression of other cellular receptors, *e.g.*, complement receptor type 1 (CR1) (Seya *et al.* 1990) and the insulin-like growth factor-II/mannose-6-phosphate receptor (O’Gorman *et al.* 1999). Generally, adhesion and/or stimulation of integrins initiate signaling events that allow cytoskeletal rearrangements, cellular migration, and immunological activation. Adhesive and cytoskeletal defects are associated with fibronectin loss on transformed cells; these defects are reasoned to be due to altered integrin function (Hynes, R. O. 1987). CR3 initiates a signaling cascade in which PI 3-kinase functions as one effector (Elemer *et al.* 1994).

One function of PI-3 kinase is activation of the Rho family of small GTPases that, in turn, activate Jun-N-terminal kinase (JNK) (Hauck *et al.* 1998; Obermeier *et al.* 1998). Effector functions of JNK include regulation of gene expression and

induction of apoptosis (Hauck *et al.* 1998). Some tumor cells express proteases most of which have been described to cleave C3 (Jurianz *et al.* 1999). Binding of C3 cleavage products (*e.g.*, iC3b) to their respective receptors (*e.g.*, CR3) could trigger multiple cellular responses, including apoptosis. Additionally, CR3 can also  
5 play a role in antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cell-mediated cytotoxicity (CDCC) (Perlmann *et al.* 1983; Ramos *et al.* 1988; Ramos *et al.* 1985; Wåhlin *et al.* 1983), which facilitate tumor killing (Becherer *et al.* 1989; Erdei *et al.* 1991). Therefore, it could be reasoned that the absence of CR3 in immortal or malignant cells might confer a survival  
10 advantage to these cells.

A number of microorganisms have adapted mechanisms not only to evade complement-mediated killing but also to pilfer C' components for their own advantage. Microorganisms that initiate infection via C' receptors frequently activate C', which subsequently results in C3 deposition on their cell surface  
15 (Hondalus *et al.* 1993). The effect of C' deposition is two-fold: 1) it allows for evasion of immune surveillance, and 2) it allows targeting to the appropriate host cell (Cooper, N. R. 1991). Microbial entry of host cells in a CR3 opsonic-dependent manner is thought to lead to a milder respiratory burst thereby promoting increased intracellular survival (Mosser *et al.* 1987; Würzner, R. 1999). Additionally,  
20 complement-mediated endocytosis occurs independently of a proinflammatory response (Caron *et al.* 1998).

Asymptomatic gonococcal urethritis develops in a small proportion of men. In contrast, fifty to sixty percent of women with gonorrhea exhibit asymptomatic infections, and seventy percent of women with disseminated gonococcal infection  
25 (DGI) lack symptoms of genital track infection (Densen *et al.* 1982). The ability of pathogenic *Neisseria* to cause the range of disease states associated with infection requires highly efficient methods of immune avoidance. Although strain specific properties have been associated with resistance to complement-mediated killing (*i.e.*, serum resistance) *in vitro*, most clinically isolated gonococci initially exhibit

serum resistance, a property that is lost with sub-culturing (Densen, P. 1989; de la Paz *et al.* 1995; Ram *et al.* 1999; Ram *et al.* 1998; Vogel *et al.* 1999).

Ram *et al.* (1998) suggest that an increased conversion of C3b to iC3b on the gonococcal surface might contribute to serum resistance *in vivo*. This idea is supported by *in vitro* studies of gonococcal infection of neutrophils where a predominance of iC3b is found on the surface of gonococci in comparison to C3b deposition (Jarvis *et al.* 1999; McQuillen *et al.* 1999; Vogel *et al.* 1999). Conversion of C3b to iC3b on the gonococcal surface would permit efficient internalization of infecting gonococci into the cervical epithelium. Standard gentamicin-resistance assays measuring gonococcal invasion of primary endo- and ectocervical cells in the presence of anti-CR3 antibodies demonstrated greater than ninety-three percent invasion inhibition with the antibody inhibitors used.

Similar studies performed previously in the inventors' laboratory, using antibody inhibitors specific for other putative gonococcal ligands such as an antibody specific for (i) CEACAM (commercially available from Santa Cruz Biotechnology Inc.); (ii) receptors for Opa proteins; (iii) E4.3 (a monoclonal antibody specific for CD46; commercially available from Santa Cruz Biotechnology Inc.); and (iv) a receptor for pilus, failed to inhibit invasion of or association with primary endo- and ectocervical cells. Additional support for a CR3-mediated mode of gonococcal invasion of the cervical epithelium is obtained from LSCM analysis of clinical biopsies derived from women with naturally acquired gonorrhea. Confirmed co-localization of gonococci with CR3 in these tissue sections provide evidence that CR3-mediated gonococcal invasion probably occurs *in vivo*. Collectively these data suggest that CR3-mediated phagocytosis may serve as the primary mode of gonococcal invasion of the cervical epithelium.

Only a small proportion of total cellular CR3 is found on the surface of resting cells (Frank *et al.* 1991; Ram *et al.* 1998). This CR3 population is relatively immobile in the plane of the cell membrane (van Kooyk *et al.* 1999) and is thought to facilitate phagocytosis triggered by other cell surface receptors, *e.g.*, CR1 and

Fcγ receptors (Frank *et al.* 1991). A mobile, intracellular CR3 store is associated with iC3b-dependent adherence (Frank *et al.* 1991). Upon activation this latent CR3 population, which resides in peroxidase-negative granules, is rapidly released, resulting in up to a ten-fold increase in CR3 surface expression (Elemer *et al.* 1994; 5 Frank *et al.* 1991; Kishimoto *et al.* 1989). Early in the stages of phagocytosis CR3 aggregation also occurs (Caron *et al.* 1998; Elemer *et al.* 1994; Frank *et al.* 1991; Kishimoto *et al.* 1989; van Kooyk *et al.* 1999). LSCM analysis of *N. gonorrhoeae* infected primary endo- and ectocervical cells were reflective of these events. Co-localization of infecting gonococci was readily visible by thirty minutes post- 10 infection of endo- and ectocervical cells, and this association became more pronounced by ninety minutes and three hours post-infection suggesting an increase in surface level expression of CR3. Additionally, co-localization of gonococci with CR3 was evident as clusters on the endo- and ectocervical cell surfaces.

Several studies have demonstrated that efficient signal transduction mediated 15 through CR3 that subsequently allows phagocytosis may require co-operation among receptors that share adherence to a particular organism (Elemer *et al.* 1994; Frank *et al.* 1991; Hayashi *et al.* 1997; Ingalls *et al.* 1998; Kishimoto *et al.* 1989; Mesri *et al.* 1998; Stocks *et al.* 1995; Stocks *et al.* 1996; Wright *et al.* 1983). Cross-linking of this/these co-receptors to CR3 is thought to induce a conformational 20 change in CR3 that leads to its increased ligand avidity and/or affinity followed by an increase in cell surface expression, a process called inside-out signaling. Studies focusing on the interaction of putative neisserial virulence factors with host cells have clearly demonstrated that the establishment of productive infection is multifactorial and several bacterial products may play a synergistic role in successful 25 invasion.

The present inventors have demonstrated a role for CR3-mediated invasion of primary endo- and ectocervical cells by the gonococcus. The mechanism used by this bacterium to achieve CR3 adherence is reported in Edwards and Apicella, 2002 and Edwards *et al.*, 2002. Anti-CR3 immunoprecipitation studies of infected,

primary endo- and ectocervical cell lysates demonstrated that gonococcal porin, pili, and opa proteins associate with CR3. These data may be indicative of opsonic (*i.e.*, iC3b-mediated) adherence, alternatively, unopsonic binding of porin, pili, and opa proteins each to either CR3 or their respective co-receptor may facilitate CR3-mediated entry. CR3 up-regulation can be blocked by neutrophil treatment with an anion-specific channel blocker, but binding of neutrophils to endothelial cells remained unaffected (Kishimoto *et al.* 1989). *N. gonorrhoeae* porin proteins are anion selective water-filled channels that are capable of transmigration to and insertion into eukaryotic cell membranes (Bjerknes *et al.* 1995; Lynch *et al.* 1984); therefore, it is possible that these proteins play a role in up-regulation of CR3 upon gonococcal attachment.

Recent data has suggested that an association with selective members of the carcinoembryonic antigen family of cell adhesion molecules (CEACAM) (Stocks *et al.* 1995; Stocks *et al.* 1996) may augment CR3 activity. CEACAM are suggested to initiate a priming signal in neutrophils that results in activation of adhesion receptors without the release of inflammatory mediators or the induction of a respiratory burst (Stocks *et al.* 1995). CEACAM1 and CEACAM5 are also present on epithelial cells and have been shown to bind gonococcal Opa. It is tempting to speculate a role for an Opa-CEACAM interaction in CR3-mediated invasion. However, previous data and unpublished work in the inventors' laboratory has demonstrated that invasion of a *N. gonorrhoeae* strain FA1090 Opa deletion mutant and strain 1291 Opa<sup>-</sup> phase variant (isolated on the basis of colony morphology) is comparable to their respective wild type counterparts. Additionally, membrane ruffling was observed upon SEM analysis of these Opa<sup>-</sup> strains. Therefore the significance of Opa proteins to these studies is unclear.

One possibility is that binding of heparin to Opa facilitates fH (which possesses three heparin-binding domains (Zipfel *et al.* 1999)) adherence to surface bound C3. Support of this idea is that Chen *et al.* (1995) demonstrated that heparin treatment of gonococci resulted in a fifty-five to eighty-five percent increase in

survival in normal human serum. fH has also been demonstrated to bind gonococcal porin. fH possesses a sialic acid binding site that has been shown to bind sialylated gonococcal LOS; consequently, the redundancy of the ability of fH to bind the gonococcus would preclude the absolute requirement for Opa proteins for successful  
5 infection by the gonococcus.

Membrane co-factor protein (CD46) serves as a C' regulatory protein on the surface of all nucleated cells thereby protecting them from C' mediated lysis. Similar to fH, CD46 functions on the cell surface as a co-factor for fI-mediated C' inactivation (Seya *et al.* 1990). CD46 has been shown to function as a receptor for  
10 gonococcus pili on unpolarized ME180 cells (Källström *et al.* 1997); however, in polarized epithelial cells CD46 exists on the basolateral surface (Maisner *et al.* 1997). Additionally, CD46 is not efficiently endocytosed and those surface molecules that are internalized are rapidly degraded (Maisner *et al.* 1997). These findings preclude the possibility of receptor recycling to the apical cell surface. The  
15 inventors' unpublished data and the work of others strongly suggests that gonococcal pili play a crucial role in gonococcal pathogenesis. A soluble form of CD46 (sCD46) also exists (Jurianz *et al.* 1999) and is present in seminal fluid (Vanderpuye *et al.* 1992); however, the significance of this molecule is unclear. In view of this work, its intriguing to speculate that the interaction of gonococcal pili  
20 with sCD46 may augment the function of CR3 possibly by binding to or near the divalent cation binding domain of CR3.

The presence of  $Mn^{2+}$  and  $Ca^{2+}$  are speculated to directly induce integrin changes required for efficient ligand binding by circumventing physiological triggering events (Altieri, D. C. 1991; Stewart *et al.* 1996; Violette *et al.* 1995).  
25 Kallstrom *et al.* (2000) recently demonstrated that adherence of non-piliated *N. gonorrhoeae* strain MS11 could be induced to occur on ME180 cells in the presence of  $Ca^{2+}$ . Although the inventors were unable to detect CR3 in any of the immortalized or malignant cell lines examined in this work (including ME180 cells), the  $Ca^{2+}$ -mediated invasion of non-piliated gonococci observed by Kallstrom

*et al.* might have occurred through an alternative integrin receptor. The cation-dependent induction of receptor function is a property attributed to integrins in general (Altieri, D. C. 1991).

SEM analysis demonstrated that the addition of anti-CR3 antibodies to  
5 CHO-CR3 and primary endo- and ectocervical cell monolayers resulted in membrane ruffles, suggesting that this phenomenon is elicited by CR3 activation. Upon gonococcal infection of primary human endo- and ectocervical cells membrane ruffling is induced to occur (Edwards *et al.* 2000). TEM analysis of clinical cervical biopsies, which were derived from women with documented  
10 gonococcal cervicitis, suggested that membrane ruffling also occurred *in vivo* (Edwards *et al.* 2000). Additionally, membrane ruffling was predominately accompanied by a concentrated accumulation of the actin-associated proteins ezrin and vinculin (Edwards *et al.* 2000).

Jones *et al.* (1998) recently described two CR3 signaling pathways: 1) FcγR-  
15 induced, PI-3 kinase dependent and 2) formylmethionylleucylphenylalanine (fMLP)-induced, PI-3 kinase independent pathways. Both modes of CR3 signaling lead to the activation of p21 activating kinase 1 (PAK1) (Jones *et al.* 1998). PAK1 is a serine/threonine kinase demonstrated to exhibit multiple effector functions. PAK1 can regulate membrane ruffling both independently and dependently of the action of  
20 Rac (Obermeier *et al.* 1998; Sells 1997). Additionally, PAK1 regulates the formation of vinculin-containing focal complexes (Obermeier *et al.* 1998; Sells 1997). The ability of PAK1 to regulate membrane ruffling and vinculin accumulation through a CR3-dependent signaling cascade corresponds well with previously described data, and data presented herein. Additionally, this supports  
25 evidence for the induction of membrane ruffling of primary, human endo- and ectocervical cells by the binding of the gonococcus to CR3.

It is interesting to note that *Shigella* are capable of membrane ruffle induction and that these organisms parasitize the rectal epithelium (Tran Van Mhieu *et al.* 1999), which also exhibits CR3 expression (Hussain *et al.* 1995). Also of



interest is that the sexually transmitted organisms, *Candida* and HIV, are both capable of CR3-mediated internalization of host cells (Cooper, N. R. 1991; Hussain *et al.* 1995; Würzner, R. 1999). The pathogenic *Neisseria* have evolved multiple efficient mechanisms by which to evade host defense mechanisms. Among these  
5 immune avoidance mechanisms are the strain-specific attributes that confer serum-resistance *e.g.*, sialylation of some LOS glycoforms and a P.1A porin serotype (Densen, P. 1989; Ram *et al.* 1999; Vogel *et al.* 1999; *et al.* 1992). *In vitro* gonococcal infection studies and examination of clinically isolated gonococci have revealed C' components (predominately iC3b) on the surface of gonococci (Densen,  
10 P. 1989; Jarvis *et al.* 1999; McQuillen *et al.* 1999; Ross *et al.* 1985). Additionally, gonococci have been demonstrated to activate both the classical and alternative C' pathways; however, gonococcal killing primarily occurs via the CP (Densen *et al.* 1982). This would suggest a role for AP inactivation (and possibly subsequent CR3-mediated internalization) as one mechanism by which the gonococcus persists  
15 within its primary niche, the human reproductive tract. The inventors' data suggests that CR3-mediated invasion serves as a primary mechanism by which *N. gonorrhoeae* invades the cervical epithelium. This process involves ruffling of the cervical epithelium, which appears to be triggered by CR3 engagement.

## 20 Vaccine Preparations

The present invention thus provides a vaccine for use to protect mammals against *Neisseria* colonization or infection, *e.g.*, *N. gonorrhoeae* and/or *N. meningitidis*. For example, the vaccine may contain an immunogenic amount of polypeptide PLD, also known as p55, from *N. gonorrhoeae*, or an immunogenic  
25 amount of polypeptide PLD from *N. meningitidis* in combination with a physiologically-acceptable, non-toxic vehicle. Vaccines of the present invention can also include effective amounts of immunological adjuvants known to enhance an immune response.

The immunogenic neisserial protein can be conjugated or linked to another peptide or to a polysaccharide. For example, immunogenic proteins well-known in the art, also known as “carriers,” may be employed. Useful immunogenic proteins include keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA),

5 ovalbumin, human serum albumin, human gamma globulin, chicken immunoglobulin G and bovine gamma globulin.

Further provided are isolated and purified nucleic acid molecules, *e.g.*, DNA molecules, comprising a nucleic acid segment that encodes at least a portion of a neisserial protein. For example, the invention provides an expression cassette  
10 comprising a DNA segment that codes for an RNA molecule that is substantially identical (sense) to all or a portion of a messenger RNA (“target” mRNA), *i.e.*, an endogenous or “native” neisserial protein mRNA. The DNA segment in the expression cassette is operably linked to a promoter. As used herein, “substantially identical” in sequence means that two nucleic acid sequences have, for example, at  
15 least about 65%, about 70%, about 90%, or about 98% contiguous nucleotide sequence identity to each other. As an example, the preselected DNA segment hybridizes under hybridization conditions, such as stringent hybridization conditions, to a nucleic acid molecule encoding the corresponding native neisserial protein.

20 As used herein, “substantially pure” means an object species is the predominant species present (*i.e.*, on a molar basis it is more abundant than any other individual species in the composition). For example, a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a  
25 substantially pure composition will comprise more than about 80 percent of all macromolecular species present in the composition, for example, more than about 85%, about 90%, about 95%, and about 99%. The object species can be purified to essential homogeneity (contaminant species cannot be detected in the composition

by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

As used herein, the term "recombinant nucleic acid" or "nucleic acid," *e.g.*, "recombinant DNA sequence or segment" refers to a nucleic acid, *e.g.*, to DNA, that has been derived or isolated from any appropriate source, that may be subsequently chemically altered *in vitro*, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in a genome that has not been transformed with exogenous DNA. An example of DNA "derived" from a source, would be a DNA sequence that is identified as a useful fragment within a given organism, and which is then chemically synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from said source by chemical means, *e.g.*, by the use of restriction endonucleases, so that it can be further manipulated, *e.g.*, amplified, for use in the invention, by the methodology of genetic engineering.

Recovery or isolation of a given fragment of DNA from a restriction digest can employ separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. See Lawn *et al.*, Nucleic Acids Res., 9, 6103 (1981), and Goeddel *et al.*, Nucleic Acids Res., 8, 4057 (1980). Therefore, "DNA" includes completely synthetic DNA sequences, semi-synthetic DNA sequences, DNA sequences isolated from biological sources, and DNA sequences derived from RNA, as well as mixtures thereof. As used herein, the term "derived" with respect to a RNA molecule means that the RNA molecule has complementary sequence identity to a particular DNA molecule.

Nucleic acid molecules encoding amino acid sequence variants of a neisserial protein are prepared by a variety of methods known in the art. These

methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the  
5 neisserial protein.

To immunize a subject, the neisserial protein is administered parenterally, usually by intramuscular or subcutaneous injection in an appropriate vehicle. Other modes of administration, however, are also acceptable. For example, the vaccine may be administered orally, or via a mucosal route, such as a nasal, gastrointestinal  
10 or genital site. Vaccine formulations will contain an effective amount of the active ingredient in a vehicle. The effective amount is sufficient to prevent, ameliorate or reduce the incidence of *N. gonorrhoeae* colonization in the target mammal. The effective amount is readily determined by one skilled in the art. The active ingredient may typically range from about 1% to about 95% (w/w) of the  
15 composition, or even higher or lower if appropriate. The quantity to be administered depends upon factors such as the age, weight and physical condition of the human subject considered for vaccination. The quantity also depends upon the capacity of the person's immune system to synthesize antibodies, and the degree of protection desired. Effective dosages can be readily established by one of ordinary skill in the  
20 art through routine trials establishing dose response curves. The subject is immunized by administration of the neisserial protein in one or more doses. Multiple doses may be administered as is required to maintain a state of immunity to streptococci.

To prepare a vaccine, the purified neisserial protein can be isolated,  
25 lyophilized and stabilized. The neisserial protein may then be adjusted to an appropriate concentration, optionally combined with a suitable vaccine adjuvant, and packaged for use. Suitable adjuvants include but are not limited to surfactants, e.g., hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N,N-dioctadecyl-N'-N-bis(2-hydroxyethyl-propane di-amine),

methoxyhexadecyl-glycerol, and pluronic polyols; polyanions, *e.g.*, pyran, dextran sulfate, poly IC, polyacrylic acid, carbopol; peptides, *e.g.*, muramyl dipeptide, MPL, aimethylglycine, tuftsin, oil emulsions, alum, and mixtures thereof. Other potential adjuvants include the B peptide subunits of *E. coli* heat labile toxin or of the cholera toxin. McGhee, J.R., *et al.*, "On vaccine development," Sem. Hematol., 30:3-15 (1993). Finally, the immunogenic product may be incorporated into liposomes for use in a vaccine formulation, or may be conjugated to proteins such as keyhole limpet hemocyanin (KLH) or human serum albumin (HSA) or other polymers.

## 10 Antibodies

The antibodies of the invention are prepared by using standard techniques. To prepare polyclonal antibodies or "antisera," an animal is inoculated with an antigen, *i.e.*, a purified immunogenic PLD peptide or polypeptide, and immunoglobulins are recovered from a fluid, such as blood serum, that contains the immunoglobulins, after the animal has had an immune response. For inoculation, the antigen is preferably bound to a carrier peptide and emulsified using a biologically suitable emulsifying agent, such as Freund's incomplete adjuvant. A variety of mammalian or avian host organisms may be used to prepare polyclonal antibodies against gonococcal or meningococcal PLD.

Following immunization, Ig is purified from the immunized bird or mammal, *e.g.*, goat, rabbit, mouse, rat, or donkey and the like. For certain applications, particularly certain pharmaceutical applications, it is preferable to obtain a composition in which the antibodies are essentially free of antibodies that do not react with the immunogen. This composition is composed virtually entirely of the high titer, monospecific, purified polyclonal antibodies to PLD, or peptides thereof. Antibodies can be purified by affinity chromatography, using purified PLD, or peptides thereof. Purification of antibodies by affinity chromatography is generally known to those skilled in the art (see, for example, U.S. Patent No. 4,533,630). Briefly, the purified antibody is contacted with the purified PLD, or

peptide thereof, bound to a solid support for a sufficient time and under appropriate conditions for the antibody to bind to the polypeptide or peptide. Such time and conditions are readily determinable by those skilled in the art. The unbound, unreacted antibody is then removed, such as by washing. The bound antibody is then recovered from the column by eluting the antibodies, so as to yield purified, monospecific polyclonal antibodies.

Monoclonal antibodies can be also prepared, using known hybridoma cell culture techniques. In general, this method involves preparing an antibody-producing fused cell line, *e.g.*, of primary spleen cells fused with a compatible continuous line of myeloma cells, and growing the fused cells either in mass culture or in an animal species, such as a murine species, from which the myeloma cell line used was derived or is compatible. Such antibodies offer many advantages in comparison to those produced by inoculation of animals, as they are highly specific and sensitive and relatively “pure” immunochemically. Immunologically active fragments of the present antibodies are also within the scope of the present invention, *e.g.*, the  $F_{(ab)}$  fragment scFv antibodies, as are partially humanized monoclonal antibodies.

Thus, it will be understood by those skilled in the art that the hybridomas herein referred to may be subject to genetic mutation or other changes while still retaining the ability to produce monoclonal antibody of the same desired specificity. The present invention encompasses mutants, other derivatives and descendants of the hybridomas.

It will be further understood by those skilled in the art that a monoclonal antibody may be subjected to the techniques of recombinant DNA technology to produce other derivative antibodies, humanized or chimeric molecules or antibody fragments that retain the specificity of the original monoclonal antibody. Such techniques may involve combining DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of the monoclonal antibody with DNA coding the constant regions, or constant regions plus framework

regions, of a different immunoglobulin, for example, to convert a mouse-derived monoclonal antibody into one having largely human immunoglobulin characteristics (see EP 184187A, 2188638A, herein incorporated by reference).

## 5 Inhibitory Compounds

The present invention provides a method of preventing entry of *Neisseria gonorrhoeae* and/or *N. meningitidis* into a cell (or treating an existing infection) by administering a compound that inhibits, *e.g.*, reduces the activity of, neisserial PLD. In particular, it has been discovered that it is possible to prevent the infection of  
10 cervical cells (endocervical or ectocervical cells) by blocking the activity of *N. gonorrhoeae* PLD. Any inhibitor could be used. For example, the inhibitor could be an antibody (*e.g.*, a monoclonal or polyclonal antibody, or a fragment of an antibody) that specifically binds to *N. gonorrhoeae* PLD, *i.e.*, *N. gonorrhoeae* PLD, or a compound such as a divalent cation chelator that inhibits gonococcal  
15 association and/or invasion or primary ectocervical cells and/or endocervical cells.

## Formulations of Compounds and Methods of Administration

In cases where compounds are sufficiently basic or acidic to form stable nontoxic acid or base salts, administration of the compounds as salts may be  
20 appropriate. Examples of pharmaceutically acceptable salts are organic acid addition salts formed with acids that form a physiological acceptable anion, for example, tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate,  $\alpha$ -ketoglutarate, and  $\alpha$ -glycerophosphate. Suitable inorganic salts may also be formed, including hydrochloride, sulfate, nitrate, bicarbonate, and  
25 carbonate salts.

Pharmaceutically acceptable salts are obtained using standard procedures well known in the art, for example by reacting a sufficiently basic compound such as an amine with a suitable acid affording a physiologically acceptable anion. Alkali

metal (for example, sodium, potassium or lithium) or alkaline earth metal (for example calcium) salts of carboxylic acids also are made.

The compounds may be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient in a variety of forms adapted to the chosen route of administration, *i.e.*, orally or parenterally, by intravenous, intramuscular, topical or subcutaneous routes.

Thus, the present compounds may be systemically administered, *e.g.*, orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the active compound may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of active compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active



compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the  
5 active compound may be incorporated into sustained-release preparations and devices.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts may be prepared in water, optionally mixed with a nontoxic surfactant.

10 Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the  
15 active ingredient that are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a  
20 polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about  
25 by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the

compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

For topical administration, the present compounds may be applied in pure form, i.e., when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid.

Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, alcohols or glycols or water-alcohol/glycol blends, in which the present compounds can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

Examples of useful dermatological compositions that can be used to deliver the compounds of the present invention to the skin are known to the art; for example, see Jacquet et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No.

4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

Useful dosages of the compounds of the present invention can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods  
5 for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Pat. No. 4,938,949.

Generally, the concentration of the compound(s) of the present invention in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%, preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition  
10 such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

The amount of the compound, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant  
15 physician or clinician.

In general, however, a suitable dose will be in the range of from about 0.5 to about 100 mg/kg, e.g., from about 10 to about 75 mg/kg of body weight per day, such as 3 to about 50 mg per kilogram body weight of the recipient per day, preferably in the range of 6 to 90 mg/kg/day, most preferably in the range of 15 to  
20 60 mg/kg/day.

The compound is conveniently administered in unit dosage form; for example, containing 5 to 1000 mg, conveniently 10 to 750 mg, most conveniently, 50 to 500 mg of active ingredient per unit dosage form.

Ideally, the active ingredient should be administered to achieve peak plasma  
25 concentrations of the active compound of from about 0.5 to about 75  $\mu$ M, preferably, about 1 to 50  $\mu$ M, most preferably, about 2 to about 30  $\mu$ M. This may be achieved, for example, by the intravenous injection of a 0.05 to 5% solution of the active ingredient, optionally in saline, or orally administered as a bolus containing about 1-100 mg of the active ingredient. Desirable blood levels may be

maintained by continuous infusion to provide about 0.01-5.0 mg/kg/hr or by intermittent infusions containing about 0.4-15 mg/kg of the active ingredient(s).

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four  
5 or more sub-doses per day. The sub-dose itself may be further divided, e.g., into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

The following examples are intended to illustrate but not limit the invention.

10

### **EXAMPLE 1**

#### **Membrane Ruffling and Cytoskeletal Rearrangements in *Neisseria gonorrhoeae***

The sexually transmitted pathogen *Neisseria gonorrhoeae*, the causative agent in gonorrhea, can infect the male and female genital tract. Studies have shown  
15 that the organism can discriminate between the sexes and uses different mechanisms for infection of men than for infection of women. During the course of female infection, it appears that the organism releases a group of proteins that initiate a process called membrane ruffling on endocervical and exocervical epithelial cells. Organisms adherent to or proximal to the ruffled area invade the epithelial cell. The  
20 gonococcus can proliferate within the intracellular environment, cause the death of the infected cell and are released. They re-enter new cells and the cycle continues until an inflammatory response ensues or the organisms spreads to the endometrium and fallopian tubes.

**Bacteria:** *N. gonorrhoeae* strains 1291, 1291-green (1291 expressing green  
25 fluorescent protein and to be described elsewhere, the plasmid pLES98 was a gift from V. Clark), FA1090, MS11-A, and MS11<sub>mk</sub>C were used in these infection studies. These strains are P<sup>+</sup> and Opa<sup>+</sup>. Strains 1291, 1291-green, MS11-A, and MS11<sub>mk</sub>C contain the pathogenicity island recently described by Dillard (Dillard, J. 1999).

### **Development of Primary Cervical Cell Culture Systems: Surgical**

biopsies were obtained from 30 pre-menopausal women undergoing hysterectomy at the University of Iowa Hospitals and Clinics (Iowa City, IA). Endocervical (proximal to the cervical os) and ectocervical (distal to the cervical os) tissue

5 biopsies were obtained in 4-6 mm<sup>2</sup> sections and further subdivided into 2-3 mm<sup>2</sup> sections. Sectioned tissues were rinsed twice for 10 min in Hanks Balanced Salt Solution (HBSS) supplemented with 1% fungizone (Irvine Scientific, Santa Ana, CA) and 1% penicillin (100 U/ml)-streptomycin (1 mg/ml). The tissue was placed with the epithelium downward on polystyrene, 35 mm tissue culture dishes (Falcon,

10 Becton Dickinson; Franklin Lake, NJ). Tissue explants were incubated in filtered airway medium (1 part Dulbecco Modified Eagle Medium, 1 part Ham's F12, 5% fetal calf serum (FCS), 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MO), 1% penicillin-streptomycin, and insulin (10 µg/ml)). After 48 h, airway medium was replaced with keratinocyte growth medium (KGM)-2 Bullet Kit

15 (Clonetics, San Diego, CA). KGM-2 was replaced every 2-3 days until near-confluence was obtained (1-2 weeks) at which time the cells were passaged as outlined below. Although variability exists among tissue samples, this process allows for an average of three passages of cell growth to fresh tissue culture dishes from a single tissue explant prior to fibroblast development, at which time tissue

20 explants were discarded.

**Cell Passage:** At near-confluent growth cells were passaged by a 5 min, 37°C incubation in HBSS-0.25% Trypsin-0.1% EDTA. Cell suspensions were collected and centrifuged at 5000 rpm for 5 min. The resulting cell pellet was rinsed in HBSS, resuspended in KGM-2, and used to seed transwell membrane systems

25 (Biocoat Cell Environments, Becton Dickinson, Bedford, MA) (to allow for polarized cell growth); glass, 8-well chamber slides (Nalge Nunc International, Naperville, IL); or human, placental collagen-coated, 12 mm glass coverslips previously placed in 24-well tissue culture dishes (Falcon). Primary cervical cells were maintained in KGM-2 until near-confluence was again obtained at which time

they were infected with *N. gonorrhoeae* as outlined below. Where applicable, cellular polarity was determined as an electrical resistance greater than  $2\text{K}\Omega/\text{cm}^2$  as measured across the cell monolayer. Infected and uninfected (*i.e.*, control) cervical cell-harboring membranes (from transwell systems) were subsequently subdivided  
5 into equal sections. Sections to be used for scanning electron microscopy (SEM) were processed while attached to the well apparatus so that the cellular orientation would be maintained. Remaining sections were removed from the well structure and subsequently processed independently for either confocal, transmission electron, or bright-field light microscopy.

10       **Infection of the Primary Cells:** *N. gonorrhoeae* cells allowed to grow overnight ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ) on GC-IsoVitaleX agar plates were harvested using a sterile swab and resuspended in sterile saline. Culture density was determined spectrophotometrically where an optical density (OD) of 1 at 600 nm was equivalent to  $10^9$  bacteria  $\text{ml}^{-1}$  of cell culture. Bacterial cells were then diluted to a  
15 concentration of  $10^7$  bacteria  $\text{ml}^{-1}$  in KGM-2 lacking gentamycin and used to infect  $10^5$  primary cervical cells (maintained as outlined above). Gonococcal infection was allowed to progress for variable time periods after which the infection was stopped by the removal of the infection medium, rinsing infected cervical cells with phosphate buffered saline (PBS), and cell fixation. Samples to be used in laser  
20 scanning confocal microscopy (LSCM) or differential interference contrast (DIC) analysis were immunolabeled directly following fixation. SEM, transmission electron microscopy (TEM), and bright field light microscopy (BFLM) samples were further processed by graded ethanol dehydration and resin (TEM) or paraffin (BFLM) embedment. Embedded samples were sectioned and immunolabeled as  
25 noted. Where indicated, the infection medium was harvested from the cervical cell monolayer and reused to infect fresh, uninfected cell cultures, which were subsequently processed for SEM analysis.

**Invasion Assays in the Presence of Inhibitors of Cytoskeletal Motility and Protein Synthesis:** Cervical cells were passed to 12 mm collagen-coated

coverslips as outlined above. Prior to infection with *N. gonorrhoeae* 1291 wild-type cells, primary cell cultures were left untreated, or they were pre-incubated with 300 nM wortmannin (Sigma), 1  $\mu$ M cytochalasin D (Sigma), or 400 mM ethylene glycol bis- (2-aminoethyl ether)-N, N, N', N' tetraacetic acid ((EGTA) Amresco, Solon, OH) for 2 h, 30 min, and 30 min, respectively, or they were pretreated with 100  $\mu$ g/ml nocodazole (Calbiochem-Novabiochem Corp., La Jolla, CA) for 1 h at 4°C followed by a 30 min incubation at 37°C. The requirement for *de novo* protein synthesis, either by the bacteria or by the primary cervical cells, was tested by pretreatment (30 min, 37°C) of the bacterial cultures or cervical cell monolayers with 4  $\mu$ g/ml chloramphenicol (Sigma) or 25  $\mu$ M cycloheximide (Calbiochem-Novabiochem Corp.), respectively. All chemical reagents used were maintained in the infection medium throughout the course of the infection. Trypan blue exclusion revealed no significant toxicity to the primary cervical cells at the indicated concentrations for each of the chemical reagents used. Infection was allowed to progress at 37°C, 5% CO<sub>2</sub>, for 1.5 h after which the medium was removed, the cells were rinsed with PBS, and then incubated with KGM-2 containing 100  $\mu$ g/ml gentamycin to kill extracellular bacteria. Post incubation the cervical cells were lysed with 0.5% saponin to release invasive bacteria. Percent invasion was determined as a function of the original inoculum and the number of colonies formed with subsequent plating of the cellular lysate. A Kruskal-Wallis ANOVA was used to determine the statistical significance of the calculated percent invasion for each of the cytoskeletal motility inhibitors used with respect to the untreated, infected cell cultures.

**Microscopy:** Samples were processed for LSCM, SEM, or TEM as previously described (Ketterer *et al.* 1999). Samples to be analyzed by BFLM were paraffin embedded using an automated tissue processor (RMC 1530 Paraffin Tissue Processor, Tucson, AZ), cut into thick (1  $\mu$ m) sections, and mounted onto glass microscope slides. Immunolabeling of infected and uninfected cervical cells for TEM analysis was performed using the monoclonal antibody 2C3, which specifically recognizes the H.8 gonococcal surface protein, or the anti-gonococcal

porin monoclonal antibody, 3H1 (a gift from Mylan Blake); in conjunction with a polyclonal antibody to filamentous (F) actin. Secondary labeling proceeded with the use of 30 nm and 10 nm colloidal gold-beaded antibody conjugates (Amersham Pharmacia Biotech, Piscataway, NJ) to the bacterial- and actin-specific antibodies, respectively. B. A. Evans generously provided clinical biopsies used in TEM analysis. The samples were viewed with an H-7000 Hitachi Transmission Electron Microscope (Hitachi Corporation, Mountain View, CA).

Primary antibodies used for LSCM or DIC microscopy were as follows: anti-cytokeratin 8.12 (Sigma), -cytokeratin 4 (Sigma), -talin (Sigma), -vinculin (Sigma), - $\alpha$ -actinin (Sigma), -myosin (Sigma), -ezrin (Santa Cruz Biotechnology, Santa Cruz, CA), -CD66 (DAKO, Carpinteria, CA), -CD46 (Santa Cruz Biotechnology), and 2C3. Immunolabeling of cervical cell monolayers with anti-cytokeratin, -talin, -vinculin, -myosin, -ezrin, and - $\alpha$ -actinin occurred subsequent to a 15 min incubation in 0.2% Triton X-100 to allow cervical cells to become permeable. Where indicated, counter staining occurred at room temperature (RT) for 6 min. Counter stains used were specific for nucleic acids and consisted of YOYO-1 (Molecular Probes, Eugene, OR) or ethidium bromide. Samples were viewed using the BioRad MRC-1024 or the Zeiss 510 Laser Scanning Confocal viewing systems.

Cervical tissue biopsies (obtained as outlined above) to be used for LSCM cytokeratin analysis were processed (within 1-2 h of obtaining the tissue specimen) for cyrosectioning by a 30 min incubation in 1% paraformaldehyde followed by infiltration with 30% sucrose prior to embedment in Tissue-Tek O. C. T. compound (Sakura Finetek USA, Inc., Torrance, CA) and sectioning (6-8 nm). Frozen sections were allowed to stand at RT for 1h prior to immunolabeling with the indicated anti-cytokeratin antibody. A fluorescein isothiocyanate (FITC)-conjugated secondary antibody was applied and tissues were subsequently counter-stained with ethidium bromide (0.5 ng/ml, 6 min).



Cervical cells passaged to 12 mm coverslips were used to assay for gonococcal-induced macropinocytosis. Cervical cell monolayers were infected with 1291-green for variable time periods in the presence of 1 mg/ml tetramethylrhodamine B isothiocyanate (TRITC)-dextran (MW 150,000). Infection was stopped by the removal of the infection medium. Infected monolayers were extensively washed prior to fixation with 2% paraformaldehyde. Coverslips were mounted onto glass microscope slides and viewed using the BioRad MRC-1024 Laser Scanning Confocal viewing system.

Slides prepared for BFLM were hematoxylin-eosin stained using a standard protocol and viewed with a Leitz Diaplan microscope with an Optronics Engineering viewing system. SEM analysis was performed using an H-4000 Hitachi Scanning Electron Microscope (Hitachi).

## Results

### 15      **Characterization of Primary Human Endocervical Epithelial Cells:**

Primary cervical epithelial cells were allowed to grow as described above. Epithelial cells could be seen extending from the cervical explants within two to three days from the start of the cultures. Growth radiated from the tissue foci in a contiguous monolayer, and confluence was observed within ten to fourteen days.

20      Transfer of endocervical-derived cells to transwell membrane systems resulted in polarized cell growth.

The cytokeratin expression pattern of the normal human uterine cervix has been well characterized. LSCM was used to determine the cytokeratin expression pattern of the primary cervical cell monolayers with respect to the tissue from which they were derived. Sectioned tissue biopsies (obtained from the endo- and ectocervix) and the cervical-derived cell monolayers were immunohistochemically examined with antibodies to cytokeratins 4, 13, 15, and 16. The results of these studies can be seen in FIG. 4. The specific cytokeratin staining character of the endo- and ectocervical tissue was retained in the primary cell monolayers (FIG. 4).

LSCM analysis of sectioned tissue biopsies and cervical-derived cell monolayers demonstrated the expression of CD66 and CD46 in both the endo- and ectocervix.

***N. gonorrhoeae* Infection of Primary Cervical Epithelial Cells: SEM**

5 analysis of *N. gonorrhoeae* 1291 infected polarized and non-polarized cells showed bacteria could adhere to both types of primary cervical cells. Bacteria were found distributed across the monolayer surface. The interaction of the bacteria with the cervical cell surface appeared to occur by multiple mechanisms. At approximately ten minutes post infection gonococci could be found associated with the cervical cell  
10 membrane both dependent (FIG. 5A) and independent of microvilli (FIG. 5B). Small tufts of microvilli were associated with bacteria on some cervical cells. Gonococci associated with the cervical cells independent of microvilli appeared to be entering the cervical cell by an endocytic process. At approximately 20 and 30 minutes post-infection, filopodia and lamellipodia formation was readily observed  
15 (FIG. 5C) and bacteria appeared to be undergoing internalization (FIG. 5D). Additionally, a visible smoothing of the cervical cell membrane was evident around the periphery of some sites of bacterial infection (FIG. 5E). By 60 minutes post-infection, the filopodia and lamellipodia became less prominent. Large membrane ruffles (FIGs 5F and 5G) became prominent at about 90 minutes post infection of  
20 cervical cells. Membrane ruffles were abutting and contiguous with gonococci. Generally, ruffles could be readily identified by a smoothing of the cervical cell membrane that encircled the ruffle (FIG. 5H). At 3 h post-gonococcal infection, membrane ruffles and bacteria associated with microvilli were still evident. Perturbations of the cell membrane that were reminiscent of ruffles were also  
25 evident. Ruffling could be induced to occur at approximately thirty minutes post-gonococcal infection in both primary cell systems when uninfected cervical cells were infected with a primed infection inoculum (*i.e.*, infection medium transferred from an immediately prior *N. gonorrhoeae* cervical cell infection) derived from one hour (ectocervical) (FIG. 5G) and ninety minute (endocervical) infections.

Bright-field light microscopy and TEM analysis of polarized endocervical cells infected with *N. gonorrhoeae* 1291 confirmed the observation made with SEM analysis (FIG. 6). Actin-filled membrane protrusions were readily observed encompassing gonococci at ninety minutes and three hours post infection. Clusters of bacteria were found breaching the superficial cervical epithelial layer; however, bacteria entered the cervical cells as single entities with each bacterium being surrounded by its own actin-lined vacuole (FIG. 6B). Consistent with SEM analysis, gonococcus-associated membrane ruffles were readily observed at 3 h post-infection by both high-powered (TEM, FIG. 6C) and low-powered (BFLM, FIG. 6D) magnification with microscopy. TEM analysis revealed that, within the host cell cytoplasm, bacteria-containing vacuoles appeared to coalesce prior to bacterial exocytosis to the subepithelial space. TEM analysis of epon-embedded, clinically-derived cervical biopsies from women naturally infected with gonococci revealed similar processes (FIG. 7). Large membrane protrusions (indicative of ruffles) (FIG. 7B) and smaller, less organized membrane structures (FIG. 7A), were readily observed. Gonococci were, again, observed to enter the cervical cells as single entities in spacious vacuoles.

Primary cell monolayers infected with gonococci in the presence of a TRITC-conjugated dextran, which would be excluded by non-macropinocytic cellular events, demonstrated that, upon invasion, gonococci reside within macropinosomes.

LSCM analysis of infection studies performed using polarized endocervical cells and ectocervical cell monolayers suggested co-localization of CD66 and CD46 with gonococci. With extended infection (*i.e.*, six hours) clustering of CD46 molecules, which was not observed to occur at earlier time points in the infection, became prevalent in response to gonococci.

#### **Cytoskeletal Changes Occur in Cervical Cells with Gonococcal**

**Infection:** Immunolabeling of *N. gonorrhoeae* infected primary cells with antibody-conjugates to actin-associated proteins confirmed that changes of the cervical cell cytoskeletal network were occurring (FIG. 8). Antibodies to talin, vinculin, ezrin,

myosin, and  $\alpha$ -actinin demonstrated a focused accumulation of these proteins, in membrane projections, at ten minutes post infection with gonococci. Membrane projections were also observed to co-localize with gonococci. This effect was most pronounced with the use of vinculin and ezrin; however, a modest accumulation of talin and  $\alpha$ -actinin was also observed to occur. Immunolabeled projections were not observed upon analysis of uninfected cervical cells.

**Gonococcal Invasion of Cervical Cells Occurs Primarily in an Actin-Dependent Manner and Does Not Require *de novo* Protein Synthesis:** Standard gentamycin-resistance assays performed with endo- and ectocervical-derived cells confirmed results obtained by BFLM and TEM analysis and the invasive nature of gonococci with respect to both the endo- and ectocervix. Gonococci were found to invade endocervical-derived cells at a proportion of 1.57% (Table 1). A slightly higher percentage (2.70%) was observed to occur with gonococcal invasion of the ectocervical-derived cells (Table 1). The inclusion of wortmannin, cytochalasin D, and EGTA in the invasion assay prohibited bacterial entry into both cell types (Table 1). Pretreatment of primary cervical cell monolayers with the microtubule-specific depolymerizing agent, nocodazole, resulted in an approximate 67% decrease in gonococcal invasion (Table 1). Chloramphenicol and cycloheximide, which inhibit gonococcal and eukaryotic cell protein synthesis (respectively), did not inhibit gonococcal invasion of the primary cervical cell monolayers (table 1).

TABLE 1. Percent invasion of *N. gonorrhoeae* 1291 in primary cervical cells

Cell treatment	Endocervical cells			Ectocervical cells		
	Mean % invasion <sup>a</sup>	Variance of the mean	<i>p</i> <sup>b</sup>	Mean % invasion <sup>a</sup>	Variance of the mean	<i>p</i> <sup>b</sup>
None	1.5517	0.3030	NA <sup>c</sup>	2.6953	1.3569	NA
Cytochalasin D	0.0358	0.0186	0.05	0.0233	0.0046	0.025
Wortmannin	0.0177	0.0180	0.05	0.0260	0.0158	0.025
EGTA	0.0431	0.0087	0.05	0.0303	0.0052	0.025
Nocodazole	ND <sup>d</sup>	ND	ND	0.9000	0.7906	0.25
Cycloheximide	ND	ND	ND	2.5601	1.8816	0.75
Chloramphenicol	ND	ND	ND	2.6688	1.9590	0.50

<sup>a</sup> The mean is the average percentage of at least three trials in which the percent invasion was determined as a function of the original inoculum and the subsequent CFU.

<sup>b</sup> *P* values given were determined using a Kruskal-Wallis  $\kappa$ -sample test of the percent gonococcal invasion determined for each cellular treatment applied to primary cervical cells in comparison to the percent gonococcal invasion of untreated, primary cervical cells.

<sup>c</sup> NA, not applicable.

<sup>d</sup> ND, not determined.

## Discussion

Primary human ecto- and endocervical epithelial cell models have been described whose cytokeratin, CD66, and CD46 profiles are identical to the tissue from which they were derived. Confocal and electron microscopic analysis of primary, human, cervical cells infected with *N. gonorrhoeae* 1291, FA1090, and MS11 have demonstrated the ability of gonococci to adhere to and to induce cytoskeletal changes within both of these cell systems. Bacteria were found to associate with the primary cervical cells by more than one mechanism as evidenced by microvillus-dependent and -independent modes of bacterial attachment. Membrane perturbations resulted in the formation of membrane ruffles, which became prominent by ninety minutes post infection and after which ruffles remained readily observable. Ruffling could be induced to occur at thirty minutes post gonococcal infection in both primary cell systems when uninfected cervical cells were infected with a primed infection inoculum; however, *de novo* protein synthesis was not required to prime the infection process for invasion. Actin-associated proteins were also observed to accumulate in response to gonococcal infection. Gonococci were found to be internalized within the cervical cells in actin-lined spacious vacuoles.

The ability of gonococci to attach to the endocervical epithelium is well accepted. In contrast, attachment to the stratified squamous epithelium of the ectocervix and to transitional cells of the cervical squamocolumnar junction (Draper *et al.* 1980; Evans, B. A. 1977) remains controversial. Studies, *in vitro*, with the inventors' primary cell culture systems demonstrated gonococcal adherence to both the endo- and ectocervix. Considerable anatomical variation exists in the length of the squamocolumnar transition zone of the cervix (Fluhmann, C. F. 1959). Additionally, to a variable measure, columnar epithelium may overlap the stratified squamous epithelium (of the ectocervix) at the transition zone. This may, in part, account for the controversy associated with gonococcal attachment to the cervical epithelium. Cervical biopsies, used in the studies described herein, were obtained

from sites distinct from the transformation zone *i.e.*, greater than 0.5 cm from the squamocolumnar junction. Of the thirty cervical specimens used to generate primary cell cultures for use in these studies, all have supported gonococcal adherence with minimal variability. Gonococcal adherence, to date, has primarily  
5 been associated with microvilli formation; however, gonococci associated with the cervical epithelium were found both dependent and independent of microvilli.

Attachment is not synonymous with tissue damage or with the initiation of a diseased state; it is a discrete event from phagocytic internalization *i.e.*, invasion. Four general mechanisms of bacterial invasion of host cells have been proposed to  
10 occur: receptor mediated endocytosis (Robinson, M. S. 1994), microtubule-dependent endocytosis (Mukherjee *et al.* 1997; Oelschlager *et al.* 1993; Silverstein *et al.* 1977), zippering (Griffin, Jr., *et al.* 1976; Griffin, Jr., *et al.* 1975), and triggering (Dramsi *et al.* 1998; Finlay *et al.* 1997; Moulder, J. W. 1985; Rabinovitch, M. 1995; Watarai *et al.* 1996). Several eukaryotic cell surface  
15 molecules have been proposed to serve as receptors for gonococcal invasion (for review Dehio *et al.* 1998; Dramsi *et al.* 1998; Jerse *et al.* 1997; McGee *et al.* 1983; Meyer, T. F. 1999; Nassif *et al.* 1999; Nassif *et al.* 1995; Naumann *et al.* 1999). In fallopian tube organ culture (FTOC) gonococcal invasion has been proposed to occur in a manner reminiscent of “zipper” type phagocytosis. (Dramsi *et al.* 1998;  
20 McGee *et al.* 1983; Stephens, D. S. 1989).

The observation that gonococci appear to induce membrane ruffling is a novel finding. Ruffling is the result of a complex interaction that occurs between a bacterium and a host cell and is associated with a triggering mechanism (Silverstein *et al.* 1977) that leads to macropinocytosis (Alpuche-Aranda *et al.*, 1994; Francis *et al.* 1993; Garcia-del Portiilo *et al.* 1994; Swanson *et al.* 1995). Infection of the  
25 inventors’ primary cell culture systems resulted in ruffling of both the endo- and ectocervical-derived cells. Ruffling was evident in the endocervical cells as convoluted spheres whereas ruffling of the ectocervical cells was observed to occur as long, ribbon-like folds. The characteristic structural morphology of endo- and



ectocervical-associated ruffles appeared to be specific for each of their respective cell types; hence, the ruffles found on the ectocervical cells were termed “ribbons.”

*Salmonella* and *Shigella* have been shown to induce membrane ruffling in a contact-dependent manner in which a (highly conserved) type III secretion system (TTSS) allows for the secretion of numerous effector proteins that initiate the cellular response required for the observed cytoskeletal rearrangements (Finlay *et al.* 1991; Rosqvist *et al.* 1995; Tran Van Mhieu *et al.* 1999). A TTSS has not been described for *N. gonorrhoeae*. A search of the *N. gonorrhoeae* strain FA 1090 genome data base (University of Oklahoma Advanced Center for Genome Technology) for the possible existence of *Salmonella* and *Shigella* TTSS and effector protein homologs yielded no significant matches to ruffling-associated proteins. Dillard *et al.* (1999) recently described the existence of a pathogenicity island in *N. gonorrhoeae* strain MS11, which encodes a secretion system. This pathogenicity island is also present in *N. gonorrhoeae* strain 1291, but it is absent in *N. gonorrhoeae* strain FA 1090. This pathogenicity island (and its encoded secretion system) may, therefore, share homology to *Salmonella* and *Shigella* TTSS and effector proteins; however, this data is currently unavailable.

Ruffling and subsequent invasion by *Salmonella* and *Shigella* shows an actin-dependence but occurs independent of microtubules. It has previously been demonstrated that gonococcal invasion of tissue culture cell lines is dependent upon microtubules and a functional actin cytoskeleton (Bessen *et al.* 1986; Grassmé *et al.* 1996; Richardson *et al.* 1998). Using standard gentamycin-resistance assays endo- and ectocervical cells were examined to determine if these primary cells displayed a microtubule- or actin-dependence for gonococcal invasion. Cytochalasin D, wortmannin, and EGTA brought invasion levels down to (essentially) zero in both cell systems suggesting that gonococcal entry is dependent upon actin rearrangements. TEM analysis of *N. gonorrhoeae* infected polarized cervical cells supported a role for actin in the gonococcal invasion process in that actin-filled ruffles and large, spacious, actin-lined vacuoles encompassed invading gonococci.

The latter finding is in contrast to Grassmé et al. (1996) who demonstrated that gonococcal association with actin was transient. In multiple experiments, using cervical cell monolayers derived from different patients, invasion was not significantly inhibited when primary cervical cells were pretreated with nocadazole  
5 to disrupt microtubules.

A concentrated accumulation of actin-associated proteins has been demonstrated to occur in response to membrane ruffling (Clerc *et al.* 1987; Finlay *et al.* 1991; Skoudy *et al.* 1999). To the knowledge of the present inventors, the role of actin-associated proteins in gonococcal infection has not been examined. It was  
10 found that in response to gonococcal invasion a concentrated accumulation of predominately ezrin and vinculin occurs in a manner analogous to *Shigella*. A modest accumulation of talin and  $\alpha$ -actinin also was observed during gonococcal infection of cervical cells. Additionally, although myosin was observed to accumulate in response to, and co-localize with, gonococci at five and ten minutes  
15 post infection, myosin was also observed to be fairly diffuse throughout some of the infected cervical cells. This may reflect the relative abundance of this protein in comparison to the other actin-associated proteins that were examined. Alternatively, the observed myosin distribution may be indicative of the initiation of a concurrent change occurring in the actin cytoskeleton, or it is possible that gonococci elicit only  
20 a minimal recruitment of myosin upon ruffle induction.

The host cell surface molecule exploited by *Salmonella* to initiate ruffling has, to date, not been elucidated. The *Shigella* protein complex of IpaB/C/D has been shown to bind the fibronectin receptor, integrin  $\alpha_5\beta_1$  (Watarai *et al.* 1996). The predominant accumulation of ezrin and vinculin in *N. gonorrhoeae* infected primary  
25 cervical cells and the ability of these actin-associated proteins to directly interact with integrin molecules to initiate cellular responses (Clarke *et al.* 1977; Schmidt *et al.* 1998) make integrin molecules attractive candidates as potential gonococcal receptors that serve to initiate gonococcal-induced ruffling. Studies using the larynx carcinoma cell line, HEP-2, have demonstrated that gonococcal binding of

fibronectin results in co-ligation of heparin sulphate proteoglycan (HSPG) to gonococcal Opa proteins and subsequent binding to the  $\alpha_5\beta_1$  integrin (Naumann *et al.* 1999). Ruffling was not observed to occur in these cells suggesting that gonococcal induction of ruffles may be unique to the cervical epithelium.

- 5 Investigation of male primary urethral cells has shown that some gonococci can enter these cells by focal macropinocytosis, but no evidence of ruffling was seen. This would suggest that perhaps a cell surface molecule unique to the cervical epithelium may be involved in ruffle induction and that gonococci invoke membrane ruffles by a mechanism distinct from that observed for *Shigella*. *Salmonella* and
- 10 *Shigella* share many common characteristics with respect to their ability to induce membrane ruffles; however, they each also display ruffling characteristics that are unique to their genus.

- Through co-evolution with their exclusive human hosts the pathogenic *Neisseria* have developed several mechanisms by which they successfully persist in
- 15 the general population. Previous studies of *N. gonorrhoeae* have demonstrated the ability of these organisms to invade eukaryotic cells by receptor-mediated endocytosis, microtubule-dependent endocytosis, and zippering. Here yet another mechanism by which gonococci are able to exploit their human host is described. Ruffling, via a triggering mechanism, has not been observed to occur in male
- 20 primary urethral cells, tissue culture cell lines, or FTOC nor has ruffling been described to occur with *Neisseria meningitidis* infections. Ruffling of primary cervical cells, which is induced with gonococcal infection, therefore, is a novel finding.

## 25 EXAMPLE 2

### **Complement Receptor 3 (CR3) Is the Factor Responsible for Ruffling**

**Tissues and Cell Culture.** Surgical biopsies derived from the endo- and the ectocervix that were used to seed primary cervical epithelial cell systems were procured and maintained as described (Example 1 above) in Defined Keratinocyte

Serum Free Medium (dk-SFM) (Life Technologies, Rockville, MD). Urethra epithelia was obtained from adult males undergoing urologic surgery at the University of Iowa Hospitals and Clinics and used to seed primary urethral cell culture systems as described by Harvey *et al.* (1997). Primary male urethral cells were immortalized with the E6 and E7 genes from the Human Papilloma Virus prior to use. E6E7 immortalized human ectocervical keratinocytes (HCK) and endocervical (End1) cells (generously provided by A. Klingehutz (University of Iowa, Iowa City, IA) and D. Anderson (Fearing Research Laboratory, Boston, MA), respectively) were cultured in dk-SFM. ME180 cervical carcinoma cells (ATCC # HTB-33) were cultured in McCoy's 5A medium (Life Technologies) according to ATCC recommendations. Hec1B endometrial carcinoma cells, Chinese hamster ovary cells (CHO-K1), and K562 myeloid cells were maintained in RPMI tissue culture medium (Life Technologies). CR3-expressing CHO (CHO-CR3) and K562 (K562-CR3) cells were maintained in RPMI-G418 (100 µg/ml). CHO cells were generously provided by L. A. Allen and L. Schlsinger (University of Iowa) with permission from D. Golenbock (Boston Medical Center, Boston, MA). E. Brown (University of Calif., San Francisco, CA) generously provided K562 and K562-CR3 cells. McCoy's 5A and RPMI media were replaced with dk-SFM 48 h prior to infection studies. Surgical biopsies derived from the fallopian tube, endometrium, endocervix, ectocervix, vas deferens, and the male and the female urethra that were to be used for immunohistochemical tissue analysis were processed for cryosectioning as previously described in Example 1 above. Clinical biopsies derived from the cervix of women with documented gonorrhea were provided by D. Fortenberry (Indiana University School of Medicine, Indianapolis, IN) and were processed for immunohistochemical analysis as previously described in Example 1 above.

**Bacteria and Infection Studies.** *N. gonorrhoeae* strains 1291, 1291-green, FA1090-green, and MS11-green were used in the infection studies described below. *N. gonorrhoeae* strains 1291-green, FA1090-green, and MS11-green express green

fluorescent protein and will be described elsewhere; the plasmid pLES98 was a gift from V. Clark (University of Rochester, Rochester, NY). *N. gonorrhoeae* 1291 and FA1090- and MS11-green parental strains (*N. gonorrhoeae* FA1090 and MS11-A, respectively) are clinically isolated gonococci. *N. gonorrhoeae* FA1090 is a serum-resistant, genital isolate from a patient with disseminated gonococcal infection. *N. gonorrhoeae* 1291 is a serum-sensitive, urethral isolate obtained from a male patient with gonococcal urethritis. *N. gonorrhoeae* 1291, 1291-green, and MS11-green contain the pathogenicity island described by Dillard *et al.* (1999). For infection studies bacteria were allowed to grow overnight (37°C, 5% CO<sub>2</sub>) on GC-IsoVitaleX agar plates prior to harvesting with a sterile swab and resuspending in sterile saline. Culture density was determined spectrophotometrically where an optical density of 1 at 600 nm was equivalent to 10<sup>9</sup> bacteria/ml. Bacterial cultures were further diluted in dk-SFM to a density of 10<sup>7</sup> bacteria/ml and used to infect cell monolayers at a multiplicity of infection of 100. Infection was allowed to progress for variable time periods after which the infection medium was removed and the cell monolayers were extensively washed with phosphate-buffered saline (PBS) prior to fixation with 2% paraformaldehyde. Uninfected, control cell monolayers were simultaneously processed with challenged cell monolayers. Infected and uninfected (control) cell monolayers were subsequently processed for Laser Scanning Confocal Microscopy (LSCM), Scanning Electron Microscopy (SEM), or Transmission Electron Microscopy (TEM) as described previously in Example 1 above; or the cells were harvested for immunoprecipitation assays.

**Immunolabeling and Microscopy.** Immunolabeling of frozen tissue sections and cell monolayers was performed as described in Example 1 above. Primary antibodies used for immunolabeling were specific for CD11b (H5A4 (Developmental Studies Hybridoma Bank (DSHB), the University of Iowa, Iowa City, IA) and Bear1 (Immunotech, Marseille, France)) or CD18 (anti-CD18 (Santa Cruz Biotechnology, Santa Cruz, CA) and IB4, generously provided by E. Brown (University of Calif.)). Tetramethyl rhodamine isothiocyanate (TRITC)- or

fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were applied to cell monolayers and bacteria, as noted. Uninfected, tissue cryosections were labeled with FITC-conjugated secondary antibodies and counter stained with ethidium bromide (0.5 ng/ml, 6 min). Clinical biopsy cryosections were incubated with 2C3 and anti-CD18 primary antibodies followed by immunolabeling with TRITC- and FITC-conjugated secondary antibodies, respectively. The 2C3 monoclonal antibody recognizes the H.8 gonococcal surface protein. Infected and uninfected (control) K562 and K562-CR3 cells to be used for TEM analysis were labeled with colloidal-gold secondary antibodies as indicated. Immunolabeled cryosections, cell monolayers, and K562 cells were viewed using the Bio-Rad MRC-1024, the Zeiss 510 Laser Scanning Confocal, or the H-7000 (Hitachi Corp., CA) transmission electron viewing systems. Primary cervical cell and CHO cell monolayers processed for SEM analyses were viewed using the Hitachi S-4000 scanning electron microscope.

**Immunoprecipitation and Western Blot Analysis.** Immunoprecipitation was performed as described by Wen *et al.* (2000). Anti-CD18 or H5A4 were used as capture antibodies. Western blotting was subsequently performed using monoclonal antibodies to gonococcal porin (3H1), pili (IE<sub>8</sub>G<sub>8</sub>), or to the opacity associated outer membrane proteins, Opa, (4B12), all of which were generously provided by M. Blake (North American Vaccine, Beltsville, MD). Antibody 6B4, which recognizes the Gal $\beta$ 1-4GlcNAc conserved epitope of gonococcal lipooligosaccharide (LOS), was used to probe for the association of LOS with CR3.

**Inhibition of *N. gonorrhoeae* Attachment and Invasion.** Primary cervical cell monolayers, CHO-CR3 and -K1 cells, and K562-CR3 and K562 cells were pretreated (30 min, 4°C) with 20  $\mu$ g/ml H5A4, Bear1, IB4, or anti-CD18 antibody competitors prior to infection with gonococci as outlined above. Where indicated anti-CD18 blocking peptide (Santa Cruz Biotechnology) was included in the inhibition assay. Infected, control cell assays (devoid of antibody competitors) and uninfected, control cell assays (with anti-CR3 antibodies) were treated in parallel

with inhibition assays. The ability of gonococci to bind primary cervical, K562-CR3, or CHO-CR3 cells in the presence or absence of antibody competitors was assessed by LSCM, TEM, or SEM qualitative analysis. Quantitative analysis of the ability of gonococci to invade primary endo- and ectocervical cells and CHO-CR3 and -K1 cells was determined by standard gentamicin-resistance assays as described previously in Example 1 above and in which antibody competitors were included or excluded from the invasion assay as described above. Where indicated primary endo- and ectocervical cell monolayers were pretreated (2h, 37°C) with 10 ng/ml *Clostridium* C3 neurotoxin prior to infection. The ability of anti-CR3 antibodies to inhibit gonococcal invasion was determined as a normalized function of the ability of gonococci to invade primary endo- and ectocervical cells and CHO cells in the absence of antibody inhibitors. A Kruskal-Wallis non-parametric analysis of variance was used to determine the statistical significance of invasion assays performed in the presence of the C3 neurotoxin.

15

## Results

**Analysis of CR3 Expression in Tissue Biopsies.** LSCM of surgical biopsies derived from the ectocervix, endocervix, endometrium, and fallopian tube revealed the presence of both the alpha and beta subunits of CR3. Immunolabeling of tissue sections with anti-CD18 and anti-CD11b (H5A4) antibodies revealed comparable levels of immunofluorescence for each antibody in each of the tissues examined. CR3 expression appeared to be greatest in the ectocervix. Expression levels decreased progressively from the ectocervix to the upper female genital tract with a low level of CR3 expression being observed in the fallopian tube tissue. Immunohistological examination of male urethra and vas deferens tissues failed to reveal the presence of either CR3 subunit. Similarly, tissue derived from the female urethra failed to label positively for CR3. An isotype control antibody yielded no immunofluorescence.

**Analysis of CR3 expression in Primary Human Cervical Epithelial Cells.** Consistent with results obtained by immunohistochemical examination of endocervical and ectocervical tissue biopsies, primary endo- and ectocervical epithelial cells labeled positive for both CD11b and CD18, and no

5 immunofluorescence was observed with an isotype control. Equivalent fluorescence was observed with either anti-CD18 or H5A4 antibodies. Immunofluorescence paralleled results obtained with immunohistological examination of tissue biopsies in that a lower level of expression was qualitatively observed in endocervical-derived cells in comparison to ectocervical-derived cells. LSCM analysis of

10 infection studies using *N. gonorrhoeae* strains 1291, 1291-green, MS11-green, and FA1090-green suggested that a higher level of CR3 surface expression occurred in the presence of the gonococcus. However, the level of CR3 expression in infected endocervical cells did not obtain that level observed for infected ectocervical cells. Infected ectocervical cells exhibited very high levels of CR3 expression. Co-

15 localization of gonococci with CR3 was observed to occur by thirty minutes post-infection; however, the gonococcus-CR3 association became more prominent by ninety minutes and three hours post-infection.

**Analysis of CR3 Expression in Immortalized Epithelial Cells.** In contrast to results obtained with primary cervical epithelial cells, cervical and endometrial

20 carcinoma cell lines (ME180 and Hec1B, respectively) failed to demonstrate CR3 expression as determined by LSCM. CR3 could not be identified on E6E7 transfected endo- and ectocervical or male urethral cells by immunofluorescence using anti-CD18 antibody or monoclonal antibody H5A4. Infection of these cell lines with gonococci revealed the presence of minimal amounts of CD18 after ninety

25 minutes and three hours; however, in comparison to results obtained with the primary cervical cells, the level of CR3 expression in the immortalized and carcinoma-derived cells was negligible. CD11b expression was not observed in ME 180, Hec1B, HCK, or End1 cells subsequent to gonococcal infection.



**Western Blot Analysis Confirmed the Presence of CR3 in Primary Cervical Cells.** To confirm the presence of CR3 in primary cervical epithelial cells immunoprecipitation was performed in which an antibody to CD11b or CD18 was used to capture CR3. Confirmation of CR3 expression was subsequently

5 demonstrated by Western Blot analysis using antibodies to CD18 or CD11b and chemiluminescence. Immunoprecipitation using the monoclonal antibody, H5A4, specific for CD11b and subsequent western blotting with anti-CD18 antibody revealed the presence of an approximately 90 kDa band consistent with CD18. The reverse experiment, in which immunoprecipitation was performed with an anti-

10 CD18 antibody and which the respective western blot was probed with H5A4, demonstrated the presence of an approximately 150 kDa band indicative of CD11b. Parallel immunoprecipitation and Western Blot experiments using male urethral epithelial cells did not reveal the presence of either CR3 subunit. Control immunoprecipitation experiments in which the H5A4 or anti-CD18 capture antibody

15 was omitted, or in which an isotype control was used as the capture antibody, failed to show the 90 or 150 kDa bands with subsequent western blotting.

**CR3 Associates with *N. gonorrhoeae* Porin, Pilus, and Opa Proteins.** To confirm LSCM analysis of gonococcal co-localization with CR3, immunoprecipitation was performed in which antibodies to CD11b or CD18 were

20 used to capture CR3 on infected and uninfected primary endo- and ectocervical cells. The association of gonococci with CR3 was subsequently examined by Western Blot analysis using antibodies to gonococcal porin, opa, or pili proteins or to LOS. Membranes probed with antibodies to LOS failed to reveal a CR3 association. Western blots probed with the monoclonal antibodies; 3H1, specific for

25 gonococcal porin, IE<sub>8</sub>G<sub>8</sub>, specific for gonococcal pili, or 4B12, which recognizes a conserved epitope of gonococcal Opa proteins, revealed that these proteins associated with CR3 present on primary endo- and ectocervical epithelial cells. Antibody probes to porin, Opa, pili, and LOS did not reveal the presence of these *N. gonorrhoeae*-associated molecules in uninfected endo- and ectocervical cells.

Immunoprecipitation (control) experiments in which the antibody to CR3 was omitted also failed to demonstrate the presence of the gonococcal-associated molecules examined.

**Anti-CR3 Antibodies Inhibit *N. gonorrhoeae* Binding to Cell Surfaces.**

5        To more closely examine the association of the gonococcus with CR3 TEM and SEM analysis was performed of the ability of *N. gonorrhoeae* to bind CR3-transformed K562 myeloid cells and CHO cells in the presence of antibodies to both the alpha and beta subunits of CR3. TEM analysis demonstrated *N. gonorrhoeae* binding to K562-CR3 cells and inhibition of *N. gonorrhoeae* binding in the presence  
10 of the anti-CR3 antibodies H5A4, Bear1, IB4, and anti-CD18. Similar results were obtained with SEM analysis of infected endo- and ectocervical cells and CHO-CR3 cells. Binding of gonococci could be inhibited by the addition of the same anti-CR3 antibodies. Binding inhibition that occurred in the presence of anti-CD18 could be reversed by the addition of the anti-CD18 blocking peptide to the infection assay.  
15 Binding of gonococci to CHO-K1 (control) cells, which do not express CR3, was not observed.

***N. gonorrhoeae* Co-localizes with CD18 *in vivo*.** The studies outlined above demonstrate that CR3 serves as a receptor for gonococcal attachment and invasion of the cervical epithelium *in vitro*. To determine if CR3 is bound by the  
20 gonococcus *in vivo*, LSCM analysis was performed of cervical biopsies derived from women with documented gonorrhea. Immunolabeling of these tissue cryosections demonstrated the presence of CD18 as a green fluorescence and gonococci as a red fluorescence. Gonococci were found to co-localize with CD18, which was visible as a yellow fluorescence. Co-localization was confirmed as a  
25 profile plot where the individual fluorescence of each fluorophore (within a designated area of presumed co-localization) was recorded and plotted, individually, by the Zeiss 510 Laser Scanning Confocal viewing system (**FIGS 9A, B**). These studies confirm *in vitro* studies using primary endo- and ectocervical cells and

provide evidence that CR3 can serve as a receptor for *N. gonorrhoeae* infection *in vivo*.

**Binding of CR3 Stimulates Membrane Ruffling.** Extensive membrane ruffling of *N. gonorrhoeae*-infected K562-CR3, CHO-CR3, and primary cervical cells was observed by TEM, SEM, and LSCM analysis. Ruffles were observed in the presence of gonococci or gonococci in the presence of anti-CR3 antibody, but membrane ruffles were not observed in uninfected cells to which antibody had not been added. Uninfected endocervical, ectocervical, and CHO-CR3 cell monolayers treated with the anti-CR3 antibodies H5A4, Bear1, IB4, and anti-CD18 also revealed extensive membrane ruffling by SEM analysis. Membrane ruffling was most pronounced with the use of the anti-CD18 antibody, IB4. Control assays using CHO-K1 cells failed to reveal the presence of membrane ruffles. These studies suggest that engagement of these cells by anti-CR3 antibodies can initiate membrane ruffling.

***N. gonorrhoeae* Invasion of Primary Endocervical and Ectocervical Cells is Dependent on CR3.** Standard gentamicin-resistance assays of infected endo- and ectocervical cells performed in the presence of antibodies to both the alpha and beta subunits of CR3 confirmed results obtained by TEM and SEM analysis of CR3-transfected myeloid and CHO cells. The addition of anti-CD11b and anti-CD18 antibodies to the invasion assays resulted in greater than 93% invasion inhibition of both endo- and ectocervical cells (**FIG. 2**) with greatest inhibition (99.86% for endocervical cells, 100% for ectocervical cells) being observed with the addition of the anti-CD11b monoclonal antibody, H5A4. Invasion inhibition that occurred in the presence of the anti-CD18 antibody could be reversed by the addition (to the invasion assay) of a blocking peptide to the anti-CD18 antibody. Pretreatment of endo- and ectocervical cells with *Clostridium* C3 neurotoxin, which inactivates the effector domain of the Rho subfamily of GTPases, also significantly inhibited gonococcal invasion supporting a role for CR3-mediated phagocytosis (**FIG. 3**).

### EXAMPLE 3

#### Identification of Inhibitory Peptides

The present inventors have a phage display library that contains 100 million different copies of 15-mer amino acids. This library is used to screen for phage particles that bind to the CR3 receptor. Briefly, the library is amplified and approximately  $10^{12}$  phage are applied to a petri dish contain CHO cells expressing CR3. The phage are allowed to interact with the cells for 1 hour and the dish is washed to remove unbound phage. The bound phage are released with a high pH (9.6 -10) buffer, reamplified and the process repeated six more times to enrich for phages particles specific for the CHO-CR3 cells. After the final enrichment, the resulting phage are placed over CHO cells lacking CR3. In this case, the unbound phage (containing CR3 binding peptides) are collected after one hour and amplified, and this process repeated six times.

At that point, enriched CR3 binding phage are plaque purified and tested for the ability to inhibit gonococcal interaction with CHO-CR3 cells. It is estimated that 100 plaque purified phages will be examined to find a phage that inhibits this interaction. When this phage is identified, the 15 mer peptide is sequenced and the peptide synthesized. Gonococcal-CHO-CR3 inhibition studies are then performed with the purified peptide.

### EXAMPLE 4

#### Radiolabeling and Collection of Gonococcal Products Released with Infection of Primary Cervical Cells.

Gonococci allowed to grow overnight on GC agar were harvested with a sterile swab and used to inoculate 5 ml cultures of Morse's Defined Medium (MDM). MDM was prepared such that half the recommended methionine and cysteine was replaced with 125  $\mu$ Ci Redivue Pro-mix L- $[^{35}\text{S}]$  *in vitro* cell labeling mix (Amersham Pharmacia Biotech Inc, Piscataway, NJ). After approximately 4 h gonococci were collected by centrifugation (4000 rpm, 5 min), rinsed with sterile

physiological saline to remove excess label, and resuspended in cold MDM such that a culture density of  $10^7$  bacteria  $\text{ml}^{-1}$  was obtained. MDM containing the  $^{35}\text{S}$ -labeled gonococci was then used to infect approximately  $10^5$  primary, human, ecto- and endocervical cells or 35 mm tissue culture dishes devoid of cervical cells. Prior to infection ecto- and endocervical cells were pretreated (30 min,  $37^\circ\text{C}$ ) with 250 mM cycloheximide to inhibit cervical cell protein synthesis. Cycloheximide was maintained in the culture medium through out the course of the infection. Cervical cells and tissue culture plates lacking cervical cells were challenged with gonococci for 90 min and 3 h time periods after which the culture supernatants were collected. Gonococci were removed from the culture supernatants by filtration through low-protein binding  $0.22\ \mu\text{m}$  syringe filter units. Supernatant filtrates were concentrated using Centricon YM-3 centrifugal filter units (Millipore Corporation, Bedford, MA) prior to suspension in 1M Tris-1% SDS. Concentrated supernatants were separated on a SDS 12% to 4% polyacrylamide gradient gel prior to gel-extraction for mass spectrometry at the Mass Spectrometry Facility located at the University of California (San Francisco, CA). Analysis of mass data was performed using Protein Prospector (University of California San Francisco, CA) (Clauser *et al.*, 1999) and ProFound (Rockefeller University, New York, NY) (Zhang *et al.*, 2000) database systems for protein identification.

During *in vitro* infection of primary endocervical and exocervical cells, the inventors have found that there is a 60 to 90 minute delay in the onset of ruffle formation after infection begins. This suggested as one possibility that the gonococcus must be releasing a factor that needed to be at a critical concentration to be effective to induce ruffling. Using bacteria labeled with  $^{35}\text{S}$  cysteine/methionine, the inventors studied the tissue culture supernatant being released by the bacteria (**FIG. 10**). Using mass spectroscopy and proteomic analysis of a SDS-Gel, the inventors identified a number of the proteins being released by the bacteria. These proteins include the following (*see*, **FIG. 11**): gonococcal protein p177, gonococcal

protein p88, gonococcal protein p64, gonococcal protein p55, gonococcal protein p46, gonococcal porin, gonococcal pilE, and gonococcal pilC.

Five of these proteins have not been previously described as important in gonococcal pathogenesis (p177, p88, p64, p55, and p46). Homologues of three of the five genes are present in the *Neisseria meningitidis* genomic database. The inventors have confirmed that the genes for two of the proteins (p177 and p55) were present in gonococcal DNA. Protein p177 encodes a 100 amino acid region that has high homology to the filamentous hemagglutinin of *Bordetella pertussis*. This protein is a bridging molecules (can span two structures) that has been shown capable of engaging and activating CR3. Protein p55 has enzymatic activity that involves modification of phospholipid membranes and could be involved in modification of the cell membrane enhancing bacterial entry.

## EXAMPLE 5

### 15                    **Inhibition of Cellular Invasion by *Neisseria gonorrhoeae***

Experiments have been performed showing that recombinant murine I-domain from the Alpha-subunit of the complement type 3 receptor inhibits *Neisseria gonorrhoeae* from invading primary human cervical cells.

The recombinant murine I-domain (rI domain) is a 23 kilodalton peptide that contains Myc and His domains. It is recognized by monoclonal antibodies specific for human CR3 I-domain. The amino acid identity to the human I domain is over 90%. The amino acid sequence of the peptide is given below (SEQ ID NO:11).

25                    FPQQESDIVFLIDGSGSINNIDFQKMKEFVSTVMEQFKKSKTLFSLMQ  
                      YSDEFRIHFTFNDFKRNPSRSHVSPKQLNGRTKTASGIRKVVRELFH  
                      KTNGARENAAKILVVITDGEKFGDPLDYKDVIPEADRAGVIRYVIGV  
                      GNAFNKPQSRRELDTIASKPAGEHVFQVDNFEALNTIQNLQEKFAL  
                      PAAASFL

The peptide is encoded by the nucleotide sequence given below (SEQ ID NO:12).

```

5  TTCCCTCAGCAGGAGAGTGACATTCTCTTCTTGATTGATGGCTCCG
   GTAGCATCAACAACATTGACTTTCAGAAGATGAAGGAGTTTGTCT
   CAACTGTGATGGAGCAGTTCAAAAAGTCTAAAACCTTGTTCTCTT
   TGATGCAGTACTCGGACGAGTTCCGGATTCACTTCACCTTCAATG
   ACTTCAAGAGAAACCCTAGCCCAAGATCACATGTGAGCCCCATA
   AAGCAGCTGAATGGGAGGACAAAAACTGCCTCAGGGATCCGGAA
   AGTAGTGAGAGAACTGTTTCACAAAACCAATGGGGCCCGGGAGA
10  ATGCTGCCAAGATCCTAGTTGTCATCACAGATGGAGAAAAATTTCG
   GTGATCCCTTGGATTATAAGGATGTCATCCCCGAGGCAFACAGAG
   CAGGGGTCATTCGCTACGTAATTGGGGTGGGAAATGCCTTCAACA
   AACCACAGTCCCGCAGAGAGCTCGACACCATCGCATCTAAGCCA
   GCTGGTGAACACGTGTTCCAAGTGGACAACCTTTGAAGCCCTGAAT
15  ACCATTCAGAACCAGCTTCAGGAAAAGATCTTTGCAATTCCCGCG
   GCCGCCAGCTTTCTA
```

Studies were performed evaluating the ability of the rI-domain to inhibit adherence and invasion of primary human ectocervical cells by *Neisseria gonorrhoeae*. The cervical cells were infected with  $10^7$  *N. gonorrhoeae* strain 1291. The results of these studies are shown in table 2 below. Recombinant I-domain is a potent inhibitor of ectocervical cell association by *Neisseria gonorrhoeae*. As little as 1 ng of rI-domain gives over 90% inhibition of invasion. With decreasing amounts of rI-domain the inhibition decreases in a dose dependent fashion.

25

**Table 2**

	<b>rI domain concentration</b>	<b>% inhibition of invasion of primary exocervical cells</b>
5	1 µg/ml	98.56
	100 ng/ml	96.8
	10 ng/ml	94.6
	1 ng/ml	92.1
	100 pg/ml	77.5
10	10 pg/ml	56.3
	1 pg/ml	17.4

Individual, smaller peptides based on the sequence of rI-domain duplicate the inhibitory activity of the rI-domain.

15

**EXAMPLE 6**

**Phospholipase D (PLD) of *Neisseria gonorrhoeae* and *Neisseria meningitidis***

Phospholipase D (PLD) is an important molecule involved in cell signaling. It hydrolyzes phosphatidylcholine (PC) to phosphatidic acid (PA) and choline in response to various extracellular stimuli. Phosphatidic acid has been implicated as a lipid second messenger to a variety of extracellular stimuli. Phosphatidic acid has been implicated as a lipid second messenger in the regulation of protein kinases, GTPase-activating proteins, PI kinases, adenylyl cyclase and other signaling molecules. Phospholipase D has been implicated in membrane trafficking and vesicular transport, in which processes, acidic phospholipids may facilitate membrane budding and/or fusion.

Two biochemically distinct phospholipase D activities have been characterized. One is dependent upon the small GTPase Arf and upon phosphatidylinositol 4, 5-bisphosphate (PIP<sub>2</sub>) and another is stimulated by oleate. Phospholipase D activation by v-Src depends upon a GTPase cascade containing Ras and Ral. Ral constitutively associates with phospholipase D through Ral's novel



amino terminus. Evidence also implicates Rho in phospholipase D activation suggesting a complex interplay of multiple small GTPases.

The present inventors have found that polypeptide p55 is neisserial phospholipase D (PLD) (FIG. 11). PLD is released from gonococci when exposed to primary human cervical epithelial cells (FIG 12). Identification of the gene encoding *N. gonorrhoeae* PLD, *e.g.*, SEQ ID NO:9, SEQ ID NO:13, SEQ ID NO:15 and SEQ ID NO:17, which encode the polypeptides SEQ ID NO:4, SEQ ID NO:14, SEQ ID NO:16 and SEQ ID NO:18, respectively, is disclosed herein. Also disclosed herein is identification of the *pld* gene from *N. meningitidis* SEQ ID NO:19 encoding the PLD polypeptide SEQ ID NO:20.

The Neisserial *pld* DNA sequences disclosed herein were compared to other Neisserial genome sequences. Analysis revealed a more than 99% identity between a *N. gonorrhoeae* 1090 *pld* nucleic acid sequence (SEQ ID NO:25) and a more than 99% identity between a *N. gonorrhoeae* 1291 *pld* nucleic acid sequence (SEQ ID NO:27) to the Neisserial genomic sequence at the University of Oklahoma's database (SEQ ID NO:26); and a 96% identity between SEQ ID NO:25 and a 97% identity between SEQ ID NO:27 to the Neisserial genomic sequence at the Sanger database (SEQ ID NO:24).

In addition, disclosed herein is the construction of a mutant in this gene in both *Neisseria gonorrhoeae* and *N. meningitidis*, which mutants do not produce the PLD enzyme (Tables 3 and 4). The mutants have been designated 1291 $\Delta$ PLD and NMB $\Delta$ PLD, respectively.

Table 3

	Ectocervical Cells	Endocervical Cells	No cervical cells
WT 1291	0.785	0.767	0.563
1291 $\Delta$ PLD	0.409	0.401	0.208
Uninfected	0.616	0.560	NA

Positive Control (H <sub>2</sub> O <sub>2</sub> )	NA	NA	0.961
Negative Control (Buffer)	NA	NA	0.187

5

Table 4

## A. Cervical Cell Lysates

	Ectocervical Cells	Endocervical Cells	No cervical cells
WT 1291	0.597	0.636	0.527
1291 $\Delta$ PLD	0.461	0.348	0.203
Uninfected	0.435	0.380	NA

## B. Infection Supernatants

	Ectocervical Sups	Endocervical Sups	No cervical cells
WT 1291	0.408	0.435	0.563
1291 $\Delta$ PLD	0.309	0.393	0.208
Uninfected	0.207	0.368	NA

## C. Assay Controls

Positive Control (H <sub>2</sub> O <sub>2</sub> )	0.781
Negative Control (Buffer)	0.217

The present inventors cloned the gene encoding PLD, i.e., *pld*, from *Neisseria gonorrhoeae* and *N. meningitidis*, and have expressed the neisserial PLD in *Escherichia coli* using the pBAD Directional TOPO Expression Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

**A. Gonococcal PLD**

Primary ecto- and endocervical cell monolayers were pretreated and maintained with cycloheximide as described in Example 1, above, to inhibit the protein synthesis of the monolayer cells. At variable times post-infection (90 minutes and 3 hours), infection supernatants were collected from gonococci-infected and uninfected cervical cell monolayers. Supernatant filtrates were concentrated

using Centricon YM-3 centrifugal filter units (Millipore) before suspension in 0.1M Tris-0.1% SDS. Concentrated supernatants were separated (18 mA) on a SDS 12% to 4% polyacrylamide gel. Secreted products were originally identified by autoradiography. Subsequent gels were stained with Coomassie Blue for mass spectroscopy analysis, which was performed at the Mass Spectrometry Facility located at the University of California (San Francisco, CA).

While gonococcal porin and pilus have been implicated in *N. gonorrhoeae* pathogenesis, PLD has not been described in the gonococcus. Gonococcal phospholipase D (FIG. 11) is of interest because it is required for complement mediated endocytosis in professional phagocytic cells.

Analysis of infection supernatants demonstrated that gonococcal products are released upon infection of cervical epithelia. Similar results are observed upon analysis of supernatants obtained from 90 minute and 3 hour infections, from ecto- and endocervical cells, and from these same cells obtained from different tissue donors (FIG. 11).

To determine if gonococcal proteins released upon cervical infection were specific to cervical cell invasion, these studies were repeated using male urethral epithelial cells (FIG. 12). Briefly, autoradiography of pulse-labeled gonococci and Coomassie-stained gels were analyzed to determine if gonococcal products are secreted in response to infection of urethral epithelial cells. Coomassie staining was performed according to standard protocols, gels were then fixed in a methanol-acetic acid solution before staining over night at room temperature. Destaining was performed with 40% methanol with frequent exchange of the destaining solution with fresh 40% methanol, as needed. Analysis revealed that while protein products are released at 90 minutes post-infection, these proteins are not present by 3 hours of infection. Collectively, these data suggest that a small basal level of gonococcal products are released constitutively and that the continued release of gonococcal products was specific to gonococcal cervicitis.

Thus, FIG. 11 and FIG. 12 show that gonococcal PLD is released preferentially in the presence of cervical epithelial cells.

### **B. Gonoccal PLD recruits CR3**

5           PLD deficient gonococci were prepared as described hereinbelow.

Both quantitative association and invasion assays, performed as described herein, demonstrate a role for gonococcal PLD in cervical cell infection (FIG. 13). PLD-deficient (when compared to wild-type) gonococci have impaired ability to adhere to and to invade primary cervical cells. The decrease in adherence observed  
10 for mutant gonococci may be indicative of the inability of these bacteria to elicit up-regulation of CR3 surface level expression. The striking decrease in the ability of these bacteria to invade cervical epithelia may suggest a role for gonococcal PLD in CR3-mediated endocytosis or engagement of an aberrant trafficking pathway once internalized, which does not permit gonococcal intracellular survival. These  
15 processes are restored when association and/or invasion assays are performed with PLD-mutant gonococci in the presence of primed wild-type supernatants (*i.e.*, supernatants collected from a previous infection using wild-type gonococci and which have been filtered to remove bacteria), which would contain secreted gonococcal PLD. The exogenous addition of PLD from *Streptomyces spp.* to  
20 association and invasion assays of *N. gonorrhoeae*  $\Delta$ PLD infected cervical cells does not compensate for the absence of gonococcal PLD. This suggests that gonococcal PLD exhibits unique effector functions in addition to sharing structural and functional properties with *Streptomyces spp.* PLD.

Methods for CR3 detection by confocal microscopy are described  
25 hereinbelow and in Edwards et al. (2001). Confocal microscopy suggests that PLD-deficient gonococci do not elicit up-regulation of CR3 surface level expression (FIG. 14). CR3 (CD18, CR3  $\beta$ -subunit) was immunolabeled with a TRITC-conjugated antibody (commercially available from Sigma and Jackson ImmunoResearch, West Grove, PA) and was visible as a red fluorescence.

Gonococci were immunolabeled with an antibody to the highly conserved outer membrane protein, H.8 (antibody 2C3). Application of a FITC-conjugated secondary antibody (commercially available from Sigma and Jackson ImmunoResearch, West Grove, PA) allowed visualization of gonococci as a green fluorescence. Co-localization of CR3 with gonococci appeared as a yellow fluorescence because of the combined signal of the two fluorophores (FIG. 15). After 3 hours of infection of primary cervical cells with wild-type gonococci, CR3 was readily visible on the cervical epithelial surface, and the vast majority of gonococci were co-localized with this receptor. In contrast, infection with PLD-mutant gonococci revealed that only a small, sub-population of cervical cells still express CR3 on their surface. Additionally, a significant proportion of mutant gonococci are not co-localized with CR3.

As described hereinbelow, primary cervical cells were seeded in 96-well microtiter plates to comparatively quantitate surface level expression of CR3. In the procedure, cells were (i) uninfected; (ii) challenged with wild-type gonococci; (iii) challenged with PLD-mutant gonococci; and/or (iv) challenged with filtered, primed (see *infra*) wild-type or PLD-mutant gonococcal supernatants. After removal of the infection supernatant, cell monolayers were rinsed, fixed and subsequently labeled with an antibody (anti-CD11b antibody H5A4; Hildreth and August, 1985) specific for the CR3 I-domain subunit followed by an HRP antibody-conjugate (commercially available from Kirkegaard & Perry Laboratories, Gaithersburg, MD) using standard ELISA protocols. Absorbance of the o-phenylenediamine dihydrochloride peroxidase substrate was determined spectrophotometrically at 490 nm (FIG. 15). These data indicate that gonococcal PLD facilitates the up-regulation of CR3 surface level expression and confirm the observations made by confocal microscopy.

FIG. 13, FIG. 14 and FIG. 15 show that gonococcal PLD enhances the invasiveness of gonococci through the recruitment of CR3.

C. Gonoccal PLD plays a role in membrane ruffling, leading to gonococcal invasion through phosphotyrosine and phosphothreonine signal transduction events

Phospholipase D activation in mammalian cells is thought to occur early in the phagocytic process, before the onset of actin reorganization. To determine if  
5 gonococcal PLD plays a role in the cytoskeletal rearrangements leading to membrane ruffling of the cervical epithelium, scanning electron microscopy (SEM) was performed as described hereinbelow and as in Ketterer et al., 1999.

SEM analysis demonstrated that aberrant cytoskeletal rearrangements occur upon infection of cervical epithelia with PLD-mutant gonococci, when compared to  
10 infection with wild-type gonococci (FIG. 16). Endocytosis mediated by CR3 requires receptor clustering. The absence of bacterial clusters in electrographs taken of mutant gonococci at 3 hours post-infection may be reflective of the inability of these bacteria to elicit up-regulation of CR3 or of their inability to initiate signaling cascades required for CR3 clustering (FIG. 16). Similarly, the absence of membrane  
15 ruffles in PLD infected cells suggests gonococcal PLD may be required to potentiate the cytoskeletal rearrangements required to form membrane ruffles. These processes are restored when assays are performed with PLD-mutant gonococci in the presence of primed supernatants as described above. No observable differences between mutant or wild-type gonococci were noted in the ability of gonococci to interact with  
20 each other or with cervical cells at earlier points of infection (*i.e.*, less than 90 minutes).

D. Signal transduction

Protein kinases (*e.g.*, tyrosine kinases and protein kinase C (PKC)) play an  
25 integral role in CR3-mediated signal transduction in professional phagocytic cells. Additionally, in eukaryotic cell systems phospholipase D activation triggers a complex signal transduction cascade. This signal transduction cascade involves PKC and protein tyrosine stimulation, as well as the activation of small G-protein binding-proteins. To determine if signal transduction initiated upon gonococcal

infection of the cervical epithelium is altered in the absence of gonococcal PLD, Western blot analysis of cervical cell lysates was performed.

Western blotting was performed using standard protocols. Cervical cell lysates were harvested at variable times post-infection (0 minutes, 5 minutes, 10 minutes, 15 minutes, 30 minutes, 45 minutes, 60 minutes, 90 minutes, two hours, 2.5 hours, 3 hours and 4 hours) and lysed in a Tris-0.1% SDS solution. Cell lysates were separated (18 mA) on a 4% to 12% gradient polyacrylamide gel and then transferred to Immobilon-P membranes (Millipore) over-night at 150 mA. Membranes were blocked 30 minutes in 0.25% bovine serum albumin (BSA)- tris buffered saline 0.5% tween-20 (TBST) at room temperature with rotation for two hours. Membranes were then incubated with an appropriate dilution of the primary antibody. The antibody solution was removed, and membranes were rinsed 5 times for ten minutes each in wash buffer (TBST). Membranes were then incubated (1 hour, room temperature with rotation) in BSA-TBST containing a 1:10,000 dilution of the appropriate HRP secondary antibody conjugate. Membranes were rinsed five times for 5-10 minutes per rinse in wash buffer. Following the final rinse, membranes were incubated in SuperSignal (Pierce) chemiluminescent substrate and allowed to stand approximately 5 minutes in the dark before exposure to BioMax MR film (Kodak).

Western blot analyses of membranes probed with antibodies specific for phosphorylated tyrosine (anti-phosphotyrosine antibody obtained from Santa Cruz) or threonine (anti-phosphothreonine antibody obtained from Kirkegaard & Perry Laboratories) target residues revealed that alternative proteins serve as kinase and phosphatase targets in the absence of gonococcal PLD. (FIG. 17). The mass of each tyrosine kinase target (Table 5) and Ser-Thr kinase target (Table 6) was determined. Masses corresponding to targets unique to their specific experimental condition are shown in regular type, bold type corresponds to peptides too close in mass to be defined as different, and italicized type corresponds to a shared target.



Table 5

<b><u>Masses of Tyrosine Kinase Targets</u></b>	
1291-Wild-Type	1291 $\Delta$ PLD
p16.7	p21.5
p22.1	p27.2
p27.8	p40.9
p30.1	
p39.3	
p47.3	
p57.2	

Table 6

<b><u>Masses of Ser-Thr Kinase Targets</u></b>	
1291-Wild-Type	1291 $\Delta$ PLD
p9.9	p22.9
p21.7	p28.2
p25.9	p39.2
p28.2	p48.6
p40.1	
p48.4	
p58.3	

Following RNA isolation and cDNA synthesis as described hereinbelow, multiplex RT-PCR was performed using the CytoXpress MPCR Kit for Human Inflammatory Genes Set-I according to manufacturer's protocol. The analysis of cytokine message levels provided further evidence that alternative signal transduction events are triggered in the absence of gonococcal PLD (FIG. 18).

The most striking differences were observed in ectocervical cells in which the absence of gonococcal PLD resulted in decreased message levels of GAPDH, TNF- $\alpha$ , IL-6, IL-8, and TGF- $\beta$  when compared to wild-type infected cells.

As shown in FIG. 18, gonococcol PLD is able to modify human cell  
5 signaling.

#### **E. NMB PLD mutants**

The *Neisseria meningitidis* B PLD mutants were prepared as described hereinbelow.

#### **F. NMB PLD augments cytokine cellular transcription**

Human PMNLs have been shown to react differentially when stimulated with pathogenic and nonpathogenic *N. meningitidis* (Kragstbjerg *et al.*, 2000).

Using multiplex RT-PCR, the present inventors detected a number of cytokine transcripts in *N. meningitidis* infected secondary bronchial epithelial cells.

15 Multiplex RT-PCR was performed using the CytoXpress MPCR Kit for Human Inflammatory Genes Set-I (Biosource). Briefly, total RNA was isolated from secondary bronchial epithelial cells infected with pathogenic *N. meningitidis* NMB and NMBcap- and the corresponding PLD mutants. PCR was set up using DNA from uninfected primary (bronchial) cells (lane 1, FIG. 19), NMB wild-type infected  
20 cells (lane 2, FIG. 19) and NMB PLD mutant infected cells (lane 3, FIG. 19), and primers for GAPDH, TNF- $\alpha$ , IL-1 $\beta$ , GM-CSF, IL-8, IL-6 and TGF- $\beta$ . Actin was used as an internal control.

Multiplex RT-PCR was performed according to the manufacturer's protocol (BioSource) following RNA isolation and cDNA synthesis as described  
25 hereinbelow. Multiplex RT-PCR analysis of cytokine message levels provided evidence that, similar to gonococcal PLD, alternative signal transduction events are triggered in the absence of meningococcal PLD in primary human bronchial cells (FIG. 19). Differences in levels of cytokine message were restricted to un-encapsulated organisms, which is consistent with these organisms' ability to

associate with and invade airway epithelia. As compared to uninfected cells or cells infected with wild-type meningococci, decreased cytokine message levels were observed in bronchial cells that were infected with PLD mutant meningococci.

5

### Example 7

By disrupting a neisserial *pld* gene, neisserial phospholipase D synthesis and/or function, *e.g.*, enzymatic activity related to PLD such as the catalysis of phospholipase D-related hydrolysis and/or phosphatidyl-transferase reactions, is reduced, *e.g.*, inhibited.

10

Table 7

Mean PLD activity in A) uninfected and 1291 wild-type and 1291 $\Delta$ PLD infected cervical cell lysates and in B) 1291 wild-type and 1291 $\Delta$ PLD gonococci. C) Assay controls

15

A.	Ectocervical Cells		Endocervical Cells	
	Mean	Variance	Mean	Variance
WT 1291	0.777	0.06	0.772	0.03
1291 $\Delta$ PLD	0.398	0.02	0.381	0.03
Uninfected	0.515	0.08	0.526	0.06

20

B.	No Cells	
	Mean	Variance
WT 1291	0.600	0.04
1291 $\Delta$ PLD	0.179	0.03

25

C.	No Cells	
	Mean	Variance
Positive Control (H <sub>2</sub> O <sub>2</sub> )	0.885	0.11

Negative Control (Buffer)	0.182	0.03
------------------------------	-------	------

### Example 8

- 5 Anti-PLD sera was generated by BioSource International (Camarillo, CA) by injecting rabbits with a peptide designed to the first HKD region of PLD (RRMHNKSFTADNRAC comprising amino acids 181-195' (SEQ ID NO:21)). The anti-PLD antibody was screened for specificity to PLD using assays well-known to the art. This anti-PLD sera was found to inhibit PLD activity and the association and invasion of cervical cells by gonococci (Table 8).

Table 8

PLD Activity in presence or absence of 1307 anti-PLD Ab or 1307 pre-bleed serum

#### A. PLD activity

	1291 WT		1291 $\Delta$ PLD		Streptomyces spp. PLD	
	mean	variance	mean	variance	mean	variance
w/ 1307 $\alpha$ -PLD Ab	0.274	0.01	0.312	0.01	0.212	0.01
w/o 1307 $\alpha$ -PLD Ab	1.255	0.01	0.154	0.02	1.364	0.01
W/ 1307 pre-bleed	1.579	0.01	0.238	0.03	1.423	0.05

Assay Controls	mean	variance
Positive Control (H <sub>2</sub> O <sub>2</sub> )	1.557	0.01
Negative Control (Buffer)	0.125	0.01

#### 25 B. Association of primary cervical cells

	1291 WT	1291 $\Delta$ PLD
--	---------	-------------------

	mean	variance	mean	variance
w/ 1307 $\alpha$ -PLD Ab	28.13	0.42	14.84	0.94
w/o 1307 $\alpha$ -PLD-AB	7.92	0.78	14.37	1.08

5 C. Invasion of primary cervical cells

	1291 WT		1291 $\Delta$ PLD	
	mean	variance	mean	variance
w/ 1307 $\alpha$ -PLD Ab	2.7272	0.12	0.349	0.02
w/o 1307 $\alpha$ -PLD-AB	0.7969	0.03	0.3581	0.03

10

**Example 9**

2,3-diphosphoglycerate (DPG) is a specific inhibitor of PLD activity and inhibits gonococcal association and invasion of primary ecto- (pex) and endocervical (pen) cells (Table 9).

15

Table 9

Association				
	Ectocervical Cells		Endocervical cells	
	Mean	Variance	Mean	Variance
WT 1291	27.72	1.44	18.12	0.66
1291+ 100 nM DPG	15.31	1.68	6.97	1.05
Invasion				
	Ectocervical Cells		Endocervical cells	
	Mean	Variance	Mean	Variance
WT 1291	2.66	0.16	1.78	0.07
1291+ 100 nM DPG	0.36	0.01	0.21	0.01

20

25

## Example 10

### Experimental Procedures

1. Cell Culture. Surgical biopsies derived from the ecto- and the endocervix that were used to seed primary cervical epithelial cell systems were procured and
- 5 maintained as described previously (Edwards et al., 2000) in Defined Keratinocyte Serum Free Medium (dk-SFM) (Life Technologies, Rockville, MD). The primary (uec) and immortal (tuec), male urethral epithelial cells used in these studies have been described (Harvey et al. 1997; Harvey et al. 2003) and were maintained according to the methods of Harvey et al. (1997, 2003).
- 10 2. Bacteria and Infection Studies. *N. gonorrhoeae* strains 1291 (Apicella, 1974; Dudas and Apicella, 1988), FA1090 (Cohen et al., 1994), and MS11 (Schoolnik et al., 1984; Segal et al., 1985) were used in the infection studies outlined below, which were performed as previously described (Edwards et al., 2000). Briefly,
- 15 overnight cultures of gonococci were harvested from GC-IsoVitaleX agar plates with a sterile swab and resuspended in sterile physiological saline. Optical density of the bacterial suspension was determined spectrophotometrically where an optical density of 1 at 600 nm was equivalent to  $10^9$  bacteria  $\text{ml}^{-1}$ .  $10^7$  gonococci were used to infect cervical cell monolayers at a multiplicity of infection of 100. Primary
- 20 cervical cells were challenged with gonococci for variable time-periods (as noted) after which the infection medium was removed, and the cell monolayers were extensively washed with phosphate-buffered saline (PBS). Uninfected control cell monolayers were simultaneously processed with challenged cell monolayers. Infected and uninfected (control) cell monolayers were subsequently harvested for cellular fractionation, quantitative association (*i.e.*, adherence and invasion) or
- 25 invasion assays, or they were processed for microscopic analyses. Alternatively (as noted), infection supernatants were harvested, immediately transferred to ice, and gonococci were then removed by filtration through a  $0.22\ \mu\text{m}$  filter. For PLD activity assays, supernatants depleted of gonococci were filtered using Centricon

YM-30 centrifugal filter units (Millipore Corporation, Bedford, MA). Protein products were then collected with an equal volume of PLD assay buffer.

*N. gonorrhoeae* strain 1291ΔPLD was constructed by the insertion of a kanamycin-resistance cassette using the EZ::TN <KAN-2> Insertion Kit (EPICENTRE, Madison, WI). Polymerase chain reaction (PCR) of full-length NgPLD, using the primer pair of 5'-GGT GGT CAT ATG ATG CAT ACA GAC CCC AAA AT- 3' (SEQ ID NO:22) and 5'-GGT GGT TGCTCT TCC GCA TAA TAA ACC TTC TTC GAT GGG CAG-3' (SEQ ID NO. 23), suggested the insertion of the kanamycin-resistance cassette within the *pld* gene, which was then confirmed by sequence analysis performed at the University of Iowa DNA Sequencing Facility (Iowa City, IA).

**3. Radiolabeling and Collection of Gonococcal Products Released with Infection of Primary Cervical Cells.** Gonococci allowed to grow overnight on GC agar were harvested with a sterile swab and used to inoculate 5 ml cultures of Morse's Defined Medium (MDM) (Morse and Barenstein, 1980). MDM was prepared such that half the recommended methionine and cysteine was replaced with 125 μCi Redivue Pro-mix L-[<sup>35</sup>S] *in vitro* cell labeling mix (Amersham Pharmacia Biotech Inc, Piscataway, NJ). After approximately 4 hours, gonococci were collected by centrifugation (4000 rpm, 5 minutes), rinsed with sterile physiological saline to remove excess label, and resuspended in cold MDM such that a culture density of 10<sup>7</sup> bacteria ml<sup>-1</sup> was obtained. MDM containing the <sup>35</sup>S-labeled gonococci was then used to infect approximately 10<sup>5</sup> primary, human, ecto- (pex) and endocervical (pen) cells or 35mm tissue culture dishes devoid of cervical cells. Alternatively, gonococci were labeled during the course of infection by a 30-minute pulse with <sup>35</sup>S-MDM at 1 hour and 2.5 hours post-infection. Before infection pex and pen cells were treated (30 minutes, 37° C) with 250 μM cycloheximide to inhibit cervical cell protein synthesis. Cycloheximide was maintained in the culture medium through out the course of the infection. Cervical cells and tissue culture plates lacking cervical cells were challenged with gonococci for 90 minutes and 3

hours after which the culture supernatants were collected. Gonococci were removed from the culture supernatants by filtration through low-protein binding 0.22  $\mu$ m syringe filter units. Supernatant filtrates were concentrated using Centricon YM-3 centrifugal filter units (Millipore) before suspension in 0.1M Tris-0.1% SDS.

- 5 Concentrated supernatants were separated (18 mA) on a SDS 12% to 4% polyacrylamide gel before autoradiography or gel-extraction for mass spectrometry at the Mass Spectrometry Facility located at the University of California (San Francisco, CA). Analysis of mass data was performed using the Protein Prospector (University of California San Francisco, CA) and the ProFound (Rockefeller) mass  
10 analysis databases.

- 4. Determination of PLD Activity.** PLD activity was accessed using the Amplex<sup>TM</sup> Red Phospholipase D Assay Kit (Molecular Probes, Eugene OR). Wild-type and PLD mutant gonococci were suspended in PLD assay buffer to a final concentration of  $10^7$  bacteria  $\text{ml}^{-1}$ , and activity was determined according to the  
15 manufacturer's protocol. Assessment of PLD activity at acidic pH was determined in a two-step assay according to the manufacturer's protocol. For the first step,  $10^8$  gonococci were suspended in PBS with the pH adjusted to 3.0, 4.5, or 6.0.

- Gonococcal suspensions were diluted 10-fold in PLD assay buffer for the second step of the reaction. Cervical cell fractions were prepared as outlined below and  
20 PLD activity was assessed at neutral pH according to the manufacturer's protocol.

- 5. Fractionation of Primary Cervical Cells.** Uninfected (control) and infected cervical cell monolayers were lysed in buffer A (50 mM tris, pH 7.5; 10 mM NaCl, 1 mM KCl; 2 mM  $\text{MgCl}_2$ , 1 mM PMSF) by scraping cervical cells from tissue culture dishes placed on ice. The cell lysate was sonicated (two bursts of 20 seconds  
25 each). Cell debris and the nuclear fraction was removed by centrifugation (750 X g, 10 minutes), and the supernatant from this spin was then subjected to filtration through a low-protein binding 0.22  $\mu$ m syringe filter to ensure removal of gonococci. Alternatively, gonococci were removed by immunoprecipitation (as described by Wen et al., 2000) using the monoclonal antibody, 2C3, which



recognizes the H.8 outer membrane protein of the pathogenic *Neisseria*. Sucrose was added to the resulting gonococcus-depleted supernatant (S1) to a final concentration of 300 mM. Ultracentrifugation (150,000 X g, 90 minutes) was then performed to produce the plasma membrane- (pellet) and cytosol (supernatant 2, S2)-enriched fractions. The membrane-enriched fraction was resuspended in PLD assay buffer (50 mM Tris, 5 mM CaCl<sub>2</sub>, pH 8.0). S2 was concentrated by filtration through Centricon YM-30 centrifugal filter units (Millipore) after which cytosolic constituents were recovered in PLD assay buffer. Where indicated, prior to infection studies primary pex and pen cell monolayers were treated with 300 nM wortmannin (Sigma) (2 hours, 37°C) or 1 µM cytochalasin D (Sigma) (30 minutes, 37°C) to inhibit macropinocytosis of gonococci. Wortmannin and cytochalasin D were maintained in cervical cell cultures during the course of infection.

**6. RNA Isolation and RT-PCR of Primary, Human, Cervical Epithelial Cells.**

Primary, pex and pen cell monolayers were challenged with *N. gonorrhoeae* 1291 or 1291ΔPLD, or they were left uninfected. After 3 hours, infection supernatants were removed and cell monolayers were extensively rinsed with PBS. Total RNA (intracellular gonococcal and cervical cell RNA) was isolated using the RNAqueous-4PCR kit (Ambion, Inc., Austin, TX) according to the manufacturer's protocol. Ribosomal RNA was removed from the total RNA using the MICROBExpress kit (Ambion) according to the manufacturer's protocol, yielding message-enriched bacterial and cervical cell RNA. Cervical cell RNA was then separated from intracellular bacterial RNA using the Poly(A)Purist kit (Ambion) according to the manufacturer's protocol with slight modification. Supernatants from the capture and wash steps, which contained gonococcal RNA, were saved and pooled. Gonococcal RNA was recovered by ethanol precipitation. cDNA was synthesized using RETROscript™ First Strand Synthesis Kit for RT-PCR (Ambion); reactions lacking the reverse transcriptase (RT) (negative control) were run simultaneously with reactions containing RT. PCR analysis of reverse-transcribed and of mock reactions using primers to α-actin and to gonococcal reduction

modifiable protein (Rmp) demonstrated the absence of contaminating DNA and of gonococcal DNA and RNA in the isolated cervical cell RNA. PCR of reverse-transcribed cervical cell RNA was performed using the primer pairs 5'-TCC ATG CAA GAA TCT GGT TTC-3' (SEQ ID NO:28) and 5'-CGA CAA TGA GCA CAG ACT CAC A-3' (SEQ ID NO:29) for human PLD1 to yield a 462 bp product and 5'-CCT TCA GGA TTC TGT CCA CAA-3' (SEQ ID NO:30) and 5'-CCT CTC TCA CAA CCA ATT CTT C-3' (SEQ ID NO:31) for human PLD2 to yield a 508 bp product.

#### **7. Determination of CR3 Surface Expression on Primary Cervical Cells.**

- and pen cells were passed to 96-well microtiter plates and allowed to grow to confluence. Cervical cells were then challenged with wild-type or PLD mutant gonococci after which the infection medium was removed and cells were rinsed thrice with PBS. Cells were fixed with 2% paraformaldehyde. Prior to immuno-analysis of CR3 surface level expression, cells were again rinsed with PBS.
- Immunoassays were then performed according to standard ELISA protocols using the H5A4 anti-CD11b (*i.e.*, CR3) primary and peroxidase-conjugated secondary antibodies. Absorbance of the o-phenylenediamine dihydrochloride peroxidase substrate was determined spectrophotometrically at 490 nm. Primary antibody was omitted from one well, and the secondary antibody was omitted from a second well, which served as a control for endogenous peroxidase activity. Where indicated 200 nM phorbol myristate (PMA), 200 nM 4- $\alpha$  phorbol, 100  $\mu$ M pervanadate, 3 mM hydrogen peroxide, or gonococci-depleted supernatants collected from wild-type or PLD mutant infection studies were included in the infection studies as outlined below.

#### **25 *Gonococcal PLD augments cytokine cellular transcription***

Alternative functional responses occur within cervical cells in the absence of gonococcal PLD. The data herein indicate that tyrosine and serine-threonine kinases play an integral role in CR3-mediated gonococcal cervical invasion. Pervanadate, a tyrosine kinase activator/phosphatase inhibitor, or PMA, an activator of the

serine-threonine kinase, PKC, were included in infection studies to determine if protein kinase activation could rescue the observed decrease in CR3 recruitment and cervical cell association and invasion, which occurs in the absence of gonococcal PLD (FIG. 21A and FIG. 21B). These data indicate that tyrosine kinase activation  
5 rescues CR3 recruitment, but it is not sufficient to allow gonococcal intracellular survival in the absence of gonococcal PLD. Similarly, PKC activation rescues CR3 recruitment and partially restores intracellular survival of gonococci.

The role for PLD in gonococcal cervicitis is multifactorial. PLD modulates signaling events upstream of PKC, which are required for gonococcal survival. PLD  
10 plays an early role in modulating signal transduction events leading to CR3 recruitment at the cervical cell surface.

**8. *N. gonorrhoeae* Attachment to and Invasion of Primary, Human, Cervical Cells.** Primary cervical cell monolayers were infected with wild-type or PLD  
15 mutant gonococci as outlined above. Variable concentrations of *Streptomyces spp.* PLD (SsPLD) (Sigma), PMA, or 4- $\alpha$  phorbol were included in association (adherence and invasion) or invasion assays, as noted. Cervical cells were preincubated (30 minutes, 37°C) with 200 nM PMA or 4- $\alpha$  phorbol before the addition of gonococci. 10 units ml<sup>-1</sup> SsPLD was added simultaneously with  
20 gonococci. Alternatively, where noted, cervical cell monolayers were pre-incubated (30 minutes, 37°C) with pervanadate, which was made by mixing 100  $\mu$ M ortho-sodium vanadate in 3 mM hydrogen peroxide. In separate assays, infection supernatants were collected from wild-type or PLD mutant-infected cervical cell monolayers, gonococci were removed by filtration through a 0.22  $\mu$ m syringe filter  
25 (*i.e.*, wild-type or mutant primed supernatants) and were added to association and/or invasion assays, as noted. The ability of gonococci to adhere to and/or invade pex and pen cells was quantitatively determined using standard gentamicin-resistance assays, performed as described previously (Edwards et al., 2000), and in which chemical or protein additives were included or excluded from the invasion assay as

described above. The total association (*i.e.*, adherence and invasion) of gonococci with pex and pen cells was quantitated by the omission of gentimycin from the above described invasion assay. Percent invasion of *N. gonorrhoeae* 1291 or 1291 $\Delta$ PLD in the presence or absence of experimental additives was determined as a function of the original inoculum and the number of colonies formed with subsequent plating of the cellular lysate. A Kruskal-Wallis non-parametric analysis of variance was used to determine the statistical significance of the association and invasion assays described above.

**9. Immunolabeling and Microscopy.** Immunolabeling of *N. gonorrhoeae* 1291 or 1291 $\Delta$ PLD infected pex cell monolayers was performed as described previously (Edwards et al., 2000). Primary antibodies used for immunolabeling were specific for the CR3 beta-subunit, CD18 (anti-CD18) (Santa Cruz Biotechnology, Santa Cruz, CA) or for the gonococcal H.8 outer membrane protein (antibody 2C3). FITC- and TRITC-conjugated secondary antibodies were applied to cell monolayers, as noted. Infected and uninfected (control) cell monolayers were viewed using the Bio-Rad MRC-1024 or the Zeiss 510 Laser Scanning Confocal viewing systems. Primary cervical cell monolayers were processed for SEM analyses (as described by Edwards et al., 2000) and viewed using the Hitachi S-4000 scanning electron microscope. All the microscopes used in these studies are located at the Central Microscopy Research Facility at the University of Iowa (Iowa City, IA).

## Results

**Gonococcal PLD Acts at Multiple Levels in Cervical Cell Infection.** Alternative functional responses occur within cervical cells in the absence of gonococcal PLD. Protein kinases (*e.g.*, tyrosine kinases and protein kinase C (PKC)) play an integral role in CR3-mediated signal transduction in professional phagocytic cells. Additionally, extensive PLD activity occurs upon CR3 ligation with iC3b-opsonized particles. PLD activation also triggers a complex signal transduction cascade involving PKC and protein tyrosine stimulation as well as the activation of small

G-proteins. Pervanadate, a potent phosphatase inhibitor/tyrosine kinase activator, or PMA, an activator of the serine-threonine kinase, PKC, were included in infection studies to determine if protein kinase activation could rescue the observed decrease in CR3 cell surface recruitment and cervical cell association and invasion by PLD mutant gonococci. The addition of pervanadate to infection studies demonstrated that tyrosine kinase activation rescues CR3 recruitment in the absence of NgPLD, but it is not sufficient to allow the intracellular survival of PLD mutant gonococci. No effect was observed in the association or invasion of wild-type or mutant gonococci with cervical cells or with CR3 cell surface recruitment with the addition of 3 mM hydrogen peroxide (negative control) to infection assays. Similarly, PKC activation by the addition of PMA to infection studies rescued CR3 recruitment to the cervical cell surface and partially restored intracellular survival of PLD mutant gonococci. The addition of PMA to uninfected cervical cell monolayers increased CR3 surface level expression to levels comparable to those observed with wild-type infected cells, suggesting PKC plays a critical role in CR3 recruitment to the cervical cell surface. The addition of 4-  $\alpha$ -phorbol, a non-activating analog of PMA, had no effect on gonococcal association with or invasion of cervical epithelia or on CR3 cell surface recruitment. Collectively, these data indicate that NgPLD exerts multiple effects on cervical epithelia during gonococcal infection.

## Discussion

Phospholipases play critical roles in many important cellular functions. All members of the PLD superfamily contain (usually) two HKD motifs, which are thought to associate to form a catalytic center. Within this superfamily, unique to PLD is the ability to catalyze a transphosphatidylation reaction in which a primary alcohol preferentially serves as a nucleophilic acceptor instead of water, resulting in the near-exclusive production of a phosphatidylalcohol (PtOH) at the expense of PA. The resulting PtOH is metabolically stable and, thus, serves as a specific indicator of PLD activity. Few bacterial PLDs have been described, although a role

for these enzymes in bacterial pathogenesis is suggested. Herein is described PLD activity in *N. gonorrhoeae*. A role for this secreted protein in gonococcal pathogenesis of cervical epithelia is demonstrated. Characteristic PLD activity (*i.e.*, removal of Cho by cleavage of the terminal phosphodiester bond of PtC) was observed in gonococcal whole cell lysates but was absent in gonococci in which *pld* was mutated by the insertion of a kan<sup>R</sup> cassette. PLD activity was observed over a pH range of 3.0 to 7.4, consistent with its ability to function as an effector protein within the lower female genital tract under normal (uninfected) or diseased states. The addition of SsPLD to association and invasion assays of *N. gonorrhoeae* PLD infected cervical cells did not compensate for the absence of NgPLD, suggesting NgPLD exhibits unique effector functions in addition to sharing structural and functional properties with SsPLD. An observed increase in PLD activity in infected pex and pen cells was attributed to NgPLD and not endogenous PLD activity. The ability of NgPLD activity to promote gonococcal invasion of primary cervical cells was inhibited in the presence of PtC and ethanol (a primary alcohol) but not 2-butanol (a secondary alcohol). These data definitively demonstrate that this gonococcal protein does indeed exhibit characteristic PLD function and argue against a role for endogenous pex or pen cell phospholipase C (PLC) activity in CR3-mediated invasion of cervical epithelia by gonococci. (A BLAST search of the *N. gonorrhoeae* and *N. meningitidis* genomic databases using sequences to several bacterial PLCs failed to reveal the presence of this enzyme in the pathogenic *Neisseria*.) Furthermore, these data indicate that the generation of PA or its catabolic products are required for CR3-mediated macropinocytosis of gonococci.

NgPLD appears to modulate cervical cell function, either directly or indirectly in a cooperative manner with host cell effector molecules, to promote the appropriate targeting of gonococci to permissive host cells (*i.e.*, CR3-expressing ecto- and endocervical cells) and to ensure their intracellular survival. The association with and invasion of primary cervical epithelia is impaired in the absence of NgPLD. CR3, the primary receptor by which gonococci invade the

cervical epithelia, is not recruited to the cervical cell surface in the absence of NgPLD. Membrane ruffling is not evident in the absence of NgPLD with extended infection. Cervical cell tyrosine kinase and PKC activation (at least partially) rescue signal transduction events occurring in the absence of NgPLD.

5           Integrin receptors are thought to not possess intrinsic enzymatic function, although they do interact with other cellular factors to transduce the signals required for effector functions. The cytoplasmic region of CR3 contains a constitutively phosphorylated serine residue (in resting professional phagocytic cells) on the CD11b $\alpha$ -subunit (Ahearn and Rosengard, 1998). The CD18 ( $\beta$ -subunit)  
10   cytoplasmic tail is not constitutively phosphorylated but does contain one tyrosine, three threonine, and four serine residues (Ahearn and Rosengard, 1998). Upon CR3 activation with phorbol esters (*e.g.*, PMA), phosphorylation of primarily serine residues occurs, but small amounts of phosphotyrosine and phosphothreonine are also observed (Ahearn and Rosengard, 1998; Gahmberg et al., 1998). Recent data  
15   suggest that a phosphorylation/dephosphorylation cycle occurs on the three contiguous threonine residues whereas serine phosphorylation remains stable (Gahmberg et al., 1998). Mutation of the three threonine residues results in diminished adhesive function, suggesting threonine phosphorylation plays a role in CR3 ligand binding (Gahmberg et al., 1998). It is currently not known if a similar  
20   phosphorylation cycle occurs on the CD18 cytoplasmic tyrosine residue. However, the protein tyrosine kinases Fyn, Lyk, Hck, and Frg are proposed to function in CR3-mediated signal transduction (Morley and Walport, 2000), suggesting that phosphorylation of the cytoplasmic tyrosine residue may also modulate CR3 function.

25           The absence of CR3 recruitment to the cervical cell surface and the ability of pervanadate to restore this phenotype in *N. gonorrhoeae* PLD infected primary cervical cells indicates that tyrosine phosphorylation is critical for CR3 recruitment to the infected cervical cell surface. Pervanadate does not stimulate CR3 surface recruitment in uninfected cells, suggesting that the pathway triggered by gonococci

that results in CR3 surface recruitment may be unique from that pathway promoting CR3 trafficking in resting cells. These data strongly suggest an early role for NgPLD in, directly or indirectly, modulating CR3 effector function possibly by initiating phosphorylation of the cytoplasmic tyrosine residue of the CR3 $\beta$ -subunit,

5 CD18. Studies using *Streptomyces chromofuscus* PLD (ScPLD) indicate that exogenous ScPLD can cause rapid choline release from vascular smooth muscle cells and can mimic endogenous PLD activity within these cells by triggering cytoskeletal rearrangements, DNA synthesis, and cell proliferation.

Lysophosphatidylcholine (LPtC), within the outer leaflet of the plasma membrane,

10 serves as the substrate for ScPLD cleavage resulting in the formation of lysophosphatidic acid (LPA), which, in turn, activates a PLC- and Rho-dependent signal transduction cascade upon LPA binding to its cognate G-protein coupled receptor. NgPLD, a secreted bacterial protein, modulates CR3 effector function in primary cervical epithelial cells.

15 In contrast to tyrosine kinase activation, PMA-stimulated PKC activation was capable of rescuing CR3 recruitment to the cervical cell surface and of partially rescuing the ability of gonococci to survive the mortal insult of gentamicin treatment. These data are consistent with the ability of PLD activity to regulate or to be regulated by kinase activity (Houle and Bourgoin, 1999; Choi et al., 2002).

20 These data also suggest that NgPLD acts at several levels during gonococcal invasion of cervical epithelia in that signal transduction events leading to CR3 recruitment to the cervical cell surface (tyrosine- and/or serine-threonine kinase dependent) are distinct from intracellular trafficking and/or signaling events promoting gonococcal survival (tyrosine kinase independent, PKC dependent).

25 Several studies have linked PLD to the generation of anti-microbial reactive oxygen species (ROS) in mammalian cells. Recent evidence indicates that Ymt promotes the survival of *Y. pestis* within the flea midgut from a cytotoxic digestion product present in blood plasma and, consequently, promotes disease transmission. Data herein indicate that the presence of a functional NgPLD is essential for the tyrosine



kinase independent, PKC-dependent survival of these bacteria within primary cervical cells and that it plays a role in gonococcal survival within urethral epithelial cells.

Reorganization of the actin cytoskeleton is the result of the activation of a  
5 complex network of signal transduction pathways involving many effector  
molecules. Bacterial, plant, and human PLDs directly bind polymeric F-actin, which  
in turn increases PLD activity. In contrast, monomeric G-actin inhibits PLD activity  
in a species-specific manner in that, *in vitro*, G-actin-induced PLD inhibition is  
twenty-fold greater for human PLD1 than it is for SsPLD. The greatest degree of  
10 inhibition occurs upon the initiation of PLD activity in the presence of G-actin; less  
inhibition is observed when G-actin is added to previously activated PLD.  
Phosphatidylinositol 4, 5-bisphosphate (PIP<sub>2</sub>) is a required co-factor in human PLD  
activity. In contrast, bacterial PLD activity does not exhibit a cofactor requirement.  
Previous data have indicated that vinculin, ezrin, and  $\alpha$ -actinin co-localize with  
15 gonococci and accumulate in focal contacts in infected primary cervical cells before  
the onset of membrane ruffling. Previous data also show that consistent with  
CR3-mediated endocytosis in professional phagocytic cells, gonococcal invasion of  
the cervical epithelium requires the activation of Rho proteins. Activation of Rho  
can cause the activation of phosphatidylinositol-4-phosphate kinase, resulting in  
20 PIP<sub>2</sub> formation. In resting cells, mammalian PLD resides in an inactive state  
because PIP<sub>2</sub>, which remains bound to actin-associated proteins (*e.g.* vinculin,  
 $\alpha$ -actinin, fodrin), is unavailable as a required co-factor. Fukami et al. (1994) have  
demonstrated that activation of Balb/c 3T3 cells with platelet-derived growth factor  
resulted in a rapid decrease in the amount of PIP<sub>2</sub> that was bound by vinculin and  
25  $\alpha$ -actinin, but which was gradually reversed over a one hour incubation.

In the absence of gonococcal PLD microvilli/filopodia were formed because  
of the negative effects of vinculin and  $\alpha$ -actinin on the availability of PIP<sub>2</sub> and  
because of the presence of a (relatively) large pool of monomeric G-actin. The  
inhibitory effect of G-actin on bacterial PLD was significantly less than human PLD,

and bacterial PLDs did not require co-factor activity for function; consequently, induction of actin polymerization may be kinetically favored and, thus, be more extensive and sustained. F-actin produced would be anticipated to stimulate directly and indirectly (by depleting intracellular levels of G-actin) both gonococcal and cervical cell PLD activity, ultimately leading to membrane ruffles. Kusner et al. (2002) have demonstrated that actin binding to PLD occurs through the conserved region of this protein, which is found in all PLDs (including NgPLD), but have also suggested that heterogeneous regions may modulate this interaction. In this regard, it is of interest that PLD homologs exhibiting the highest similarity to NgPLD are found in other bacterial species (*i.e.*, *Salmonella*, *Shigella*, *Escherichia*) capable of eliciting extensive cytoskeletal rearrangements in their respective target cells.

### Example 11

#### **Gonococcal Phospholipase D Modulates the Expression and Function of Complement Receptor 3 in Primary Cervical Epithelial Cells**

Complement receptor 3 (CR3)-mediated endocytosis is a primary mechanism by which *N. gonorrhoeae* elicits membrane ruffling and cellular invasion of the cervical epithelia. Data disclosed herein indicate that, upon infection of cervical epithelia, *N. gonorrhoeae* specifically release proteins, including a phospholipase D (PLD) homolog, which facilitate membrane ruffling. To elucidate the function of gonococcal PLD in infection of the cervical epithelia, a *N. gonorrhoeae* PLD mutant was constructed. By comparative association and/or invasion assays, the PLD mutant gonococci were found to be impaired in their ability to adhere to and to invade primary cervical cells. This defect was rescued by the addition of supernatants obtained from wildtype-infected cell monolayers, but not by exogenously added *Streptomyces* PLD. The decreased level of total cell association (*i.e.*, adherence and invasion) observed for mutant gonococci is, in part, attributed to the inability of these bacteria to recruit CR3 to the cervical cell surface with extended infection. Using electron microscopy, it was demonstrated that

gonococcal PLD may be necessary to potentiate membrane ruffling and clustering of gonococci on the cervical cell surface. Data herein indicate that PLD augments CR3-mediated gonococcus invasion of, and survival within, cervical epithelia.

## Introduction

5        *Neisseria gonorrhoeae* is a strict human pathogen causing the sexually transmitted disease gonorrhea. *N. gonorrhoeae* possesses multiple mechanisms by which it is able to colonize its human host and which are dependent upon the particular microenvironment of the infection site. In this respect, the gonococcus is unique in that it senses its particular microenvironment and adjusts its mode of pathogenicity accordingly. Several gonococcal constituents have been implicated in its pathogenicity including lipooligosaccharide (LOS), porin, pilus, and the opacity-associated (Opa) outer membrane proteins. Invasion of male urethral epithelial cells is mediated by LOS, the terminal galactose of which serves as a ligand for the asialoglycoprotein receptor (ASGP-R) (Harvey et al., 2001). An intimate association between the gonococcal and host cell membranes precedes clathrin-dependent endocytosis (Harvey et al., 2001). In contrast, complement (C) receptor type 3 (CR3)-mediated endocytosis is a primary mechanism by which *N. gonorrhoeae* invade primary human cervical epithelial cells (Edwards et al., 2001). This process is dependent upon the cooperative binding of (gonococcal-bound, host-derived) iC3b, gonococcal porin, and gonococcal pilus to the I-domain of CR3 (Edwards and Apicella, 2002; Edwards et al., 2002). Engagement of CR3 results in membrane ruffling (Edwards et al., 2001) and internalization of gonococci in macropinosomes (Edwards et al., 2000).

25        Ruffling induced by gonococci during cervical cell infection is delayed from the onset of infection by 60 to 90 minutes (Edwards et al., 2000). The onset of ruffling can be accelerated to 30 minutes by the addition of filtered, pre-conditioned (*i.e.*, derived from a previous infection) media. Factors responsible for expediting the cytoskeletal changes induced by gonococcal infection were sought. Herein is disclosed the identification of gonococcal phospholipase D (PLD), which is

specifically released upon infection of cervical epithelial. Gonococcal PLD (NgPLD) was found to play a role in membrane ruffling, CR3 recruitment to the cervical cell surface, and, consequently, in gonococcal invasion of the cervical epithelia. This secreted protein is a novel, neisserial virulence factor, capable of  
5 modulating effector functions within host cells.

## Experimental Procedures

**Cell Culture.** Surgical biopsies derived from the ecto- and the endocervix that were used to seed primary cervical epithelial cell systems were procured and maintained  
10 as described previously (Edwards et al., 2000) in Defined Keratinocyte Serum Free Medium (dk-SFM) (Life Technologies, Rockville, MD). The primary (uec) (Harvey et al., 1997) and immortal (tuec) (Harvey et al., 2002), male urethral epithelial cells used in these studies have been described and were maintained according to the methods of Harvey *et al.* (Harvey et al., 2001; Harvey et al., 1997).

15 Pharmacological agents used, as described in the studies outlined below, were not cytotoxic at the indicated concentrations as determined by trypan blue exclusion.

**Bacteria and Infection Studies.** *N. gonorrhoeae* strains 1291 (Apicella, 1974; Dudas and Apicella, 1988), FA1090 (Cohen et al., 1994), and MS11 (Schoolnik et al., 1984; Segal et al., 1985) were used in the infection studies outlined below,  
20 which were performed as previously described (Edwards et al., 2000). Briefly, overnight cultures of gonococci were harvested from GC-IsoVitalX agar plates and suspended in sterile physiological saline. Optical density of the bacterial suspension was determined spectrophotometrically where an optical density of 1 at 600 nm was equivalent to  $10^9$  bacteria  $\text{ml}^{-1}$ .  $10^7$  gonococci were used to infect cervical cell  
25 monolayers at a multiplicity of infection of 100. Primary cervical cells were challenged with gonococci for variable time-periods (as noted) after which the infection medium was removed, and the cell monolayers were extensively washed with phosphate-buffered saline (PBS). Uninfected control cell monolayers were simultaneously processed with challenged cell monolayers. Infected and uninfected

(control) cell monolayers were subsequently harvested for cellular fractionation, quantitative association (*i.e.*, adherence and invasion) or invasion assays, or they were processed for microscopic analyses. Alternatively (as noted), infection supernatants were harvested, immediately transferred to ice, and gonococci were removed by filtration through a 0.22  $\mu$ m low protein-binding syringe filter. For PLD activity assays, supernatants depleted of gonococci were filtered using Centricon YM-30 centrifugal filter units (Millipore Corporation, Bedford, MA). Protein products were then collected with an equal volume of PLD assay buffer.

*N. gonorrhoeae* strain 1291 $\Delta$ PLD was constructed by the insertion of a kanamycin-resistance cassette using the EZ::TN <KAN-2> Insertion Kit (EPICENTRE, Madison, WI). Polymerase chain reaction (PCR) of full-length NgPLD, using the primer pair of 5'-GGT GGT CAT ATG ATG CAT ACA GAC CCC AAA AT- 3' (SEQ ID NO:22) and 5'-GGT GGT TGCTCT TCC GCA TAA TAA ACC TTC TTC GAT GGG CAG-3' (SEQ ID NO:23), suggested the insertion of the kanamycin-resistance cassette within the *pld* gene, which was then confirmed by sequence analysis performed at the University of Iowa DNA Sequencing Facility (Iowa City, IA).

**Radiolabeling and Collection of Gonococcal Products Released with Infection of Primary Cervical Cells.** Gonococci allowed to grow overnight on GC agar were harvested with a sterile swab and used to inoculate 5 ml cultures of Morse's Defined Medium (MDM) (Morse and Barenstein, 1980). MDM was prepared such that half the recommended methionine and cysteine was replaced with 125  $\mu$ Ci Redivue Pro-mix L-[<sup>35</sup>S] *in vitro* cell labeling mix (Amersham Pharmacia Biotech Inc, Piscataway, NJ). After approximately 4 hours, gonococci were collected by centrifugation (4000 rpm, 5 minutes), rinsed with sterile physiological saline to remove excess label, and resuspended in cold MDM such that a culture density of 10<sup>7</sup> bacteria ml<sup>-1</sup> was obtained. MDM containing the <sup>35</sup>S-labeled gonococci was then used to infect approximately 10<sup>5</sup> primary, human, ecto- (pex) and endocervical (pen) cells or 35mm tissue culture dishes devoid of cervical cells. Alternatively,

gonococci were labeled during the course of infection by a 30-minute pulse with  $^{35}\text{S}$ -MDM at 1 hour and 2.5 hours post-infection. Before infection pex and pen cells were treated (30 minutes,  $37^\circ\text{C}$ ) with  $250\text{ }\mu\text{M}$  cycloheximide to inhibit cervical cell protein synthesis. Cycloheximide was maintained in the culture medium through  
5 out the course of the infection. Cervical cells and tissue culture plates lacking cervical cells were challenged with gonococci for 90 minutes and 3 hours after which the culture supernatants were collected. Gonococci were removed from the culture supernatants by filtration through low-protein binding  $0.22\text{ }\mu\text{m}$  syringe filter units. Supernatant filtrates were concentrated using Centricon YM-3 centrifugal  
10 filter units (Millipore) before suspension in  $0.1\text{M}$  Tris- $0.1\%$  SDS. Concentrated supernatants were separated on a SDS  $4\%$  to  $12\%$  polyacrylamide gel before autoradiography or gel-extraction for mass spectrometry at the Mass Spectrometry Facility located at the University of California (San Francisco, CA). Analysis of mass data was performed using the Protein Prospector (University of California San  
15 Francisco, CA) and the ProFound (Rockefeller University, NY) mass analysis databases.

**Western Blot Analysis.** Infection supernatants depleted of gonococci (as described above) were separated on  $4\%$  to  $12\%$  denaturing polyacrylamide gradient gels and transferred to Immobilon-P membranes (Millipore). Membranes were incubated (2  
20 hours,  $37^\circ\text{C}$ ) with  $50\text{ }\mu\text{U/ml}$  neuraminidase (Roche Diagnostics, Indianapolis, IN) prior to immunodetection. Western blotting was subsequently performed according to standard protocols using the anti-lipooligosaccharide (LOS) monoclonal antibody, 6B4. This antibody recognizes the conserved  $\text{Gal}(\beta 1-4)\text{GlcNac}$  epitope of gonococcal LOS. Chemiluminescent detection was used to visualize labeled LOS.

25 **Determination of PLD Activity.** PLD activity was accessed using the Amplex™ Red Phospholipase D Assay Kit (Molecular Probes, Eugene OR). Wild-type and PLD mutant gonococci were suspended in PLD assay buffer to a final concentration of  $10^7$  bacteria  $\text{ml}^{-1}$ , and activity was determined according to the manufacturer's protocol. Assessment of gonococcal PLD activity at acidic pH was determined in a

two-step assay according to the manufacturer's protocol. For the first step,  $10^8$  gonococci were suspended in PBS with the pH adjusted to 3.0, 4.5, or 6.0.

Gonococcal suspensions were diluted 10-fold in PLD assay buffer for the second step of the reaction. Cervical cell fractions were prepared as outlined below and

- 5 PLD activity was assessed at neutral pH according to the manufacturer's protocol.

**Fractionation of Primary Cervical Cells.** Uninfected (control) and infected cervical cell monolayers were lysed in buffer A (50 mM tris, pH 7.5; 10 mM NaCl, 1 mM KCl; 2 mM  $MgCl_2$ , 1 mM PMSF) by scraping cervical cells from tissue culture dishes placed on ice. The cell lysate was sonicated (two bursts of 20 seconds

- 10 each). Cells that did not lyse and the nuclear fraction were removed by centrifugation (750 X g, 10 minutes), and the supernatant from this spin was then subjected to filtration through a low-protein binding 0.22  $\mu$ m syringe filter to ensure removal of gonococci. Sucrose was added to the resulting gonococcus-depleted supernatant (S1) to a final concentration of 300 mM. Ultracentrifugation (150,000  
15 X g, 90 minutes) was then performed to produce the plasma membrane- (pellet) and cytosol (supernatant 2, S2)-enriched fractions. The membrane-enriched fraction was resuspended in PLD assay buffer (50 mM Tris, 5 mM  $CaCl_2$ , pH 8.0). S2 was concentrated by filtration through Centricon YM-30 centrifugal filter units (Millipore) after which cytosolic constituents were recovered in PLD assay buffer.

- 20 Where indicated, prior to infection studies, primary pex and pen cell monolayers were treated with 300 nM wortmannin (Sigma) (2 hours, 37°C) or 1  $\mu$ M cytochalasin D (Sigma) (30 minutes, 37° C) to inhibit macropinocytosis of gonococci. Wortmannin and cytochalasin D were maintained in cervical cell cultures during the course of infection.

- 25 **RNA Isolation and RT-PCR of Primary, Human, Cervical Epithelial Cells.**

Primary, pex and pen cell monolayers were challenged with *N. gonorrhoeae* 1291 or 1291 $\Delta$ PLD, or they were left uninfected. After 3 hours, infection supernatants were removed and cell monolayers were extensively rinsed with PBS. Total RNA (intracellular gonococcal and cervical cell RNA) was isolated using the

RNAqueous-4PCR kit (Ambion, Inc., Austin, TX) according to the manufacturer's protocol. Ribosomal RNA was removed from the total RNA using the MICROBExpress kit (Ambion) according to the manufacturer's protocol, yielding message-enriched bacterial and cervical cell RNA. Cervical cell RNA was then  
5 separated from intracellular bacterial RNA using the Poly(A)Purist kit (Ambion) according to the manufacturer's protocol with slight modification. Supernatants from the capture and wash steps, which contained gonococcal RNA, were saved and pooled. Gonococcal RNA was recovered by ethanol precipitation. cDNA was synthesized using RETROscript™ First Strand Synthesis Kit for RT-PCR (Ambion);  
10 reactions lacking the reverse transcriptase (RT) (negative control) were run simultaneously with reactions containing RT. PCR analysis of reverse-transcribed and of mock reactions using primers to  $\beta$ -actin and to gonococcal reduction modifiable protein (Rmp) demonstrated the absence of contaminating DNA and of gonococcal DNA and RNA in the isolated cervical cell RNA. PCR of  
15 reverse-transcribed cervical cell RNA was performed using the primer pairs 5'-TCC ATG CAA GAA TCT GGT TTC-3' (SEQ ID NO:28) and 5'-CGA CAA TGA GCA CAG ACT CAC A-3' (SEQ ID NO:29) for human PLD1 to yield a 462 bp product and 5'-CCT TCA GGA TTC TGT CCA CAA-3' (SEQ ID NO:30) and 5'-CCT CTC TCA CAA CCA ATT CTT C-3' (SEQ ID NO:31) for human PLD2 to yield a 508  
20 bp product.

**Determination of CR3 Surface Expression on Primary Cervical Cells.** Pex and pen cells were passed to 96-well microtiter plates and allowed to grow to confluence. Cervical cells were then challenged with wild-type or PLD mutant gonococci after which the infection medium was removed and cells were rinsed  
25 thrice with PBS. Cells were fixed with 2% paraformaldehyde. Prior to immuno-analysis of CR3 surface level expression, cells were again rinsed with PBS. Immunoassays were then performed according to standard ELISA protocols using the H5A4 anti-CD11b (*i.e.*, CR3) primary and peroxidase-conjugated secondary antibodies. Absorbance of the o-phenylenediamine dihydrochloride peroxidase



substrate was determined spectrophotometrically at 495 nm. Primary antibody was omitted from one well, and the secondary antibody was omitted from a second well, which served as controls for non-specific binding and endogenous peroxidase activity, respectively. Where indicated gonococci-depleted supernatants collected  
5 from wild-type or PLD mutant infection studies were included in the infection studies, performed as outlined below.

***N. gonorrhoeae* Attachment to and Invasion of Primary, Human, Cervical Cells.** Primary cervical cell monolayers were infected with wild-type or PLD mutant gonococci as outlined above. Variable concentrations of PtC (Sigma),  
10 ethanol, 2-butanol, or *Streptomyces* spp. PLD (SsPLD) (Sigma) were included in association (adherence and invasion) or invasion assays, as noted. PtC, ethanol, 2-butanol, or 10 units ml<sup>-1</sup> SsPLD were added simultaneously with gonococci. In separate assays, infection supernatants were collected from wild-type or PLD mutant-infected cervical cell monolayers, gonococci were removed by filtration  
15 through a 0.22 µm syringe filter (to yield primed wild-type or mutant supernatants), which were then added to association and/or invasion assays, as noted. The ability of gonococci to adhere to and/or invade pex and pen cells was quantitatively determined using standard gentimycin-resistance assays, performed as described previously (Edwards et al., 2000) and in which chemical or protein additives were  
20 included or excluded from the invasion assay as described above. The total association (*i.e.*, adherence and invasion) of gonococci with pex and pen cells was quantitated by the omission of gentimycin from the above described invasion assay. Percent invasion of *N. gonorrhoeae* 1291 or 1291ΔPLD in the presence or absence of experimental additives was determined as a function of the original inoculum and  
25 the number of colonies formed with subsequent plating of the cellular lysate. Inhibition of gonococcal attachment and/or invasion by exogenous PtC, ethanol, or 2-butanol was determined as a normalized function of the ability of gonococci to attach to and/or invade primary cervical cells in the absence of the competitor inhibitor. A Kruskal-Wallis non-parametric analysis of variance was used to

determine the statistical significance of the association and invasion assays described above.

**Immunolabeling and Microscopy.** Immunolabeling of *N. gonorrhoeae* 1291 or 1291ΔPLD infected pex cell monolayers was performed as described previously (Edwards et al., 2000). Primary antibodies used for immunolabeling were specific for the CR3  $\beta$ -subunit, CD18 (anti-CD18 CTB104 (Santa Cruz Biotechnology, Santa Cruz, CA)) or for the gonococcal H.8 outer membrane protein (antibody 2C3). FITC- and TRITC-conjugated secondary antibodies were applied to cell monolayers, as noted. Infected and uninfected (control) cell monolayers were viewed using the Bio-Rad MRC-1024 Laser Scanning Confocal viewing system. Primary cervical cell monolayers were processed for SEM analyses (Edwards et al., 2000) and viewed using the Hitachi S-4000 scanning electron microscope. All the microscopes used in these studies are located at the Central Microscopy Research Facility at the University of Iowa (Iowa City, IA).

## 15 **Results**

***N. gonorrhoeae* Specifically Release Protein Products upon Infection of Cervical Epithelia.** Previous studies show that membrane ruffling of *N. gonorrhoeae*-infected primary human cervical cells occurs by approximately 90 minutes post-infection (Edwards et al., 2000). These same studies also demonstrate that the onset of membrane ruffling in response to gonococcal infection can be expedited by the use of a primed infection inoculum, that is, an inoculum derived from a previous infection. Based on these studies, it was reasoned that gonococcal products were being released upon infection that facilitated membrane ruffling. Autoradiography of infection supernatants demonstrated that gonococcal products were, in fact, being released with infection of primary, human, ecto- and endocervical epithelium. Release of these gonococcal products was not strain specific in that an identical protein pattern was observed with autoradiography of infection supernatants obtained from *N. gonorrhoeae* strains 1291- (FIG. 22), FA1090- (data not shown), or MS11-infected primary cervical cells (data not

shown). In contrast, analysis of supernatants derived from an identical time course of infection of uec revealed that only a small amount of gonococcal products are released by 90 minute post-infection, and by 3 hours of infection no products could be detected (FIG. 22). Collectively, these data suggested that a small basal level of gonococcal products are released constitutively, but, also that the continued release of gonococcal products was specific to gonococcal cervicitis. Western Blot analysis of infection supernatants using the anti-LOS 6B4 antibody probe did not reveal the presence of gonococcal LOS. This indicated the release of gonococcal products with cervical cell infection was the result of bacterial secretion and not of bacterial lysis (FIG. 22).

**Characterization of a *N. gonorrhoeae* PLD Homolog.** Using mass spectroscopy a subset of gonococcal products that are secreted upon cervical cell infection were identified. One secreted product, p55, was identified by mass analysis using Protein Prospector and Profound data bases as sharing significant homology to a *Neisseria meningitidis* hypothetical PLD homolog. Primers for PCR were designed based on the *N. meningitidis* serogroup B sequence and used to amplify *pld* from *N. gonorrhoeae* strains 1291, FA1090, and MS11. Sequences obtained from cloning of the PCR amplicons were then used in a BLAST search of the *N. gonorrhoeae* genome database to identify p55 as a *N. gonorrhoeae* PLD homolog (GenBank accession number AY307929). The p55 sequence was used to perform a BLAST search of the National Center for Biotechnology Information (NCBI) database, which revealed significant sequence homology of p55 to *Neisseria meningitidis* serogroups A and B hypothetical PLD homologs, hypothetical and/or putative synthases of *Escherichia coli* and *Shigella flexneri*, and a putative phospholipase of *Salmonella*. Further sequence analysis of p55 revealed that this protein contains two HKD (amino acids 184-201 and 422-439) motifs, which are required for PLD activity, and two regions of hydrophobicity (amino acids 24-34 and 217-231) that might serve as lipid association domains. Comparative assessment of PLD activity in *N. gonorrhoeae* strains 1291 and 1291 $\Delta$ PLD (performed at pH 7.4) demonstrated

PLD activity in wild-type but not mutant gonococci (Table 10), indicating p55 functions as a phospholipase D. In separate assays, NgPLD exhibited characteristic PLD activity at pH 3.0, 4.5, and 6.0 (Table 10), consistent with the capability of this enzyme to function within the lower female genital tract under normal conditions or during bacterial vaginosis and/or cervicitis.

Table 10. PLD activity in *N. gonorrhoeae* cell lysates.

PLD Activity in Gonococci				
Fluorescence Units [Mean (variance)]				
	pH 7.4	pH 6.0	pH 4.5	pH 3.0
WT 1291	0.999 (0.12)	1.489 (0.01)	1.516 (0.06)	1.537 (0.04)
1291ΔPLD	0.172 (0.03)	0.097 (0.01)	0.101 (0.01)	0.110 (0.01)
Positive control	0.930 (0.12)	1.525 (0.03)	1.468 (0.02)	1.470 (0.01)
Negative control	0.195 (0.01)	0.096 (0.01)	0.089 (0.01)	0.110 (0.01)

PLD activity was determined as described in the text. Values given are the mean values of three trials.

#### ***N. gonorrhoeae* PLD Augments Gonococcal Infection of Cervical Epithelial**

**Cells.** To determine if NgPLD plays a role in infection of cervical epithelia, quantitative association and/or invasion assays were performed using *N. gonorrhoeae* strains 1291 and 1291ΔPLD. Association and invasion assays demonstrated a role for NgPLD in gonococcal cervicitis as indicated by the decreased levels of association and of invasion observed with infection of primary cervical cells with the PLD mutant upon comparison to the wild-type bacteria (Table 11). The addition of SsPLD to association and/or invasion assays performed using mutant gonococci could not rescue the decreased levels of association and of invasion observed in the absence of NgPLD. Although efforts to isolate NgPLD have, to date, been unsuccessful, the addition of primed wild-type supernatants to infection assays performed using mutant gonococci restored association and

invasion to near-wildtype levels. However, the addition of primed mutant supernatants had no effect on the ability of PLD mutant gonococci to adhere to or to invade primary cervical cells (Table 11).

Table 11.

- 5 Percent adherence to and/or invasion of primary cervical cells by gonococci in the presence or absence of primed media or exogenous PLD.

10

A.	Ectocervical Cells		Endocervical Cells	
	Association (%)	Invasion (%)	Association (%)	Invasion (%)
<b>1291 wt</b>	28.6625 +/- 1.35	2.9086 +/- 0.22	16.7904 +/- 0.54	1.5907 +/- 0.02
<b>1291ΔPLD</b>	15.9325 +/- 0.80 (<0.05)	0.3496 +/- 0.04 (<0.05)	6.9787 +/- 0.83 (<0.05)	0.2378 +/- 0.02 (<0.05)
<b>1291ΔPLD + wt sup</b>	23.9946 +/- 3.06 (<0.05)	2.0890 +/- 0.15 (<0.05)	11.9021 +/- 1.28 (<0.05)	1.3444 +/- 0.21 (<0.05)

15

B.	Ectocervical Cells		Endocervical Cells	
	Association (%)	Invasion (%)	Association (%)	Invasion (%)
<b>1291 wt</b>	27.7631 +/- 0.14	2.6521 +/- 0.04	15.5504 +/- 0.80	1.6408 +/- 0.12
<b>1291ΔPLD</b>	15.3918 +/- 0.99 (<0.05)	0.3200 +/- 0.02 (<0.05)	6.8529 +/- 0.67 (<0.05)	0.2421 +/- 0.01 (<0.05)

<b>1291ΔPLD</b>	15.6207	0.3087	6.3615	0.2379
<b>+ pld sup</b>	+/- 0.87	+/- 0.03	+/- 0.57	+/- 0.02
	(<0.05)	(<0.05)	(<0.05)	(<0.05)

5	C.	Ectocervical Cells		Endocervical Cells	
		Association	Invasion	Association	Invasion
		(%)	(%)	(%)	(%)
	<b>1291 wt</b>	30.6436	2.9106	17.0786	1.6367
		+/- 1.69	+/- 0.39	+/- 1.43	+/- 0.11
	<b>1291ΔPLD</b>	13.6266	0.3671	7.2765	0.2009
		+/- 0.63	+/- 0.03	+/- 0.19	+/- 0.03
		(<0.05)	(<0.05)	(<0.05)	(<0.05)
	<b>1291ΔPLD</b>	14.6986	0.3862	6.8087	0.1748
	<b>+ SsPLD</b>	+/- 2.16	+/- 0.05	+/- 0.41	+/- 0.01
		(<0.05)	(<0.05)	(<0.05)	(<0.05)

- 10 Values given are the mean values in which the percent total association (adherence and/or invasion) and the percent invasion were determined as a function of the original inoculum and the subsequent number of colony forming units formed with subsequent plating of the ecto- or endocervical cell lysates. Data given are the mean values obtained from at least three trials performed in triplicate. *p*-values (noted
- 15 parenthetically) were determined using a Kruskal-Wallis k-sample analysis of variance calculated for association and/or invasion of wild-type or mutant gonococci in the presence of wild-type (A) or PLD mutant (B) primed medium or 10 U/ml SsPLD (C) in comparison to the absence of primed medium or exogenous SsPLD, as outlines in the text.
- 20

Similar studies in which tuec were challenged with *N. gonorrhoeae* strains 1291 or 1291ΔPLD revealed NgPLD does not play a role in the association of gonococci with the urethral epithelium but may promote the intracellular survival of these organisms. The association of 1291ΔPLD with tuec was comparable to that of

wild-type gonococci; whereas, invasion levels were decreased in the absence of NgPLD (Table 12).

Table 12.

Percent adherence to and/or invasion of urethral epithelial cells by wild-type and  
5 PLD mutant gonococci

	Association (%)	Invasion (%)
<b>1291 wt</b>	25.1601 +/- 1.69	2.4285 +/- 0.05
<b>1291ΔPLD</b>	25.1132 +/- 1.01 (>0.75)	1.1616 +/- 0.11 (<0.05)

10 Values given are the mean values in which the percent total association (adherence and/or invasion) and the percent invasion were determined as a function of the original inoculum and the subsequent number of colony forming units formed with subsequent plating of TUEC lysates. Data given are the mean values obtained from at least three trials performed in triplicate. *p*-values (noted parenthetically) were  
15 determined using a Kruskal-Wallis k-sample analysis of variance calculated for association and/or invasion of mutant gonococci in comparison to wild-type gonococci.

#### ***N. gonorrhoeae* PLD Plays a Role in CR3 recruitment to the Cervical Cell**

**Surface.** CR3 serves as the primary receptor for gonococcal adherence to and

20 invasion of the cervical epithelium (Edwards et al., 2001). Previous studies have also indicated that surface levels of CR3 increase with gonococcal infection (Edwards et al., 2001). Laser scanning confocal microscopy (LSCM) was performed to examine the gonococcus-CR3 association in mutant gonococci to determine if the decrease in gonococcal cervical cell association observed with use  
25 of the PLD mutant was because of the inability of mutant gonococci to recruit CR3 to the cervical cell surface. LSCM revealed that in comparison to wild-type infected pex cells, which exhibited abundant CR3 on the monolayer surface, pex cells infected with PLD mutant gonococci exhibited decreased fluorescence, indicative of a decreased level of CR3 on their cell surface (data not shown). To quantitate these

findings an ELISA assay was developed to measure cervical cell surface expression of CR3 in uninfected pex and pen cells and cells challenged with *N. gonorrhoeae* strains 1291 and 1291ΔPLD (Table 13). Immuno-analysis of the presence of CR3 on the surface of pex and pen cells confirmed our LSCM data. The amount of CR3 present on the surface of wild-type infected cervical cells was significantly greater than levels of CR3 measured on either the PLD mutant infected or uninfected cervical cells. The addition of primed wild-type supernatants to PLD mutant infected and uninfected cells increased CR3 recruitment to the cervical cell surface. However, the addition of primed supernatants from the PLD mutant had no effect on CR3 recruitment to the cervical cell surface of PLD mutant infected or uninfected cells.

Table 13

Semi-quantitative immuno-analysis of CR3 expression on the surface of primary cervical cells

	Absorbance (490 nm)	
	Ectocervical Cells	Endocervical Cells
Uninfected control	0.531 +/- 0.13	0.303 +/- 0.03
WT 1291	2.367 +/- 0.18	1.648 +/- 0.17
1291ΔPLD	0.677 +/- 0.12	0.340 +/- 0.11
Uninfected control w/ primed wt sup	1.083 +/- 0.36	0.731 +/- 0.06
Uninfected control w/ primed pld sup	0.441 +/- 0.14	0.378 +/- 0.05
1291ΔPLD w/ primed wt sup	1.364 +/- 0.43	1.131 +/- 0.28



<b>1291ΔPLD</b>	0.387	0.305
<b>w/ primed pld sup</b>	+/- 0.13	+/- 0.08
<b>No primary Ab</b>	0.063	0.083
	+/- 0.01	+/- 0.01

5 Values given are the mean values in which the presence of CR3 on the cervical cell surface was measured by an immuno-assay using the monoclonal antibody H5A4 as outlined in the text.

### ***N. gonorrhoeae* PLD Plays a Role in Membrane Ruffling of the Cervical**

**Epithelium.** PLD activation in mammalian cells is thought to occur early in the  
10 phagocytic process, before the onset of actin reorganization. To determine if gonococcal PLD plays a role in the cytoskeletal rearrangements leading to membrane ruffling of the cervical epithelium, scanning electron microscopy (SEM) was performed. SEM analysis demonstrated that aberrant cytoskeletal rearrangements occur upon infection of cervical epithelia with PLD-mutant  
15 gonococci, when compared to infection with wild-type gonococci. At 15 minutes post-infection, no significant difference was observed between PLD mutant and wild-type infected pex cells (data not shown). Small bacterial clusters were evident as were microvilli/filopodia. However, by 3 hours post-infection, bacterial clusters and membrane ruffles were not readily evident on cell monolayers infected with  
20 mutant gonococci, but were characteristically prevalent on wild-type infected cell monolayers (FIG. 16). The addition of primed wild-type supernatants, but not primed PLD mutant supernatants, to *N. gonorrhoeae* 1291ΔPLD infection studies restored bacterial clustering and membrane ruffling (FIG. 16), suggesting NgPLD plays a role in signal transduction events leading to CR3 clustering and membrane  
25 ruffling.

### **Activity and Subcellular Localization of *N. gonorrhoeae* PLD in Cervical**

**Epithelia.** To determine if PLD activity is increased in infected cervical cells, PLD activity was measured in infected and uninfected cervical cell lysates. Comparison of wild-type infected cervical cells to that of the PLD mutant infected or uninfected

cells demonstrated that overall PLD activity is increased in wild-type infected pex and pen cells. There was no significant difference between uninfected cells and mutant infected cervical cells, suggesting that the observed increase in PLD activity is primarily due to gonococcal PLD. RT-PCR analysis of human PLD1 and PLD2

5 in pex and pen cells demonstrated that endogenous cervical cell PLD message levels are not up-regulated in cells infected with *N. gonorrhoeae* strains 1291 and 1291ΔPLD when compared to uninfected cells (FIG. 23). These data support a role for NgPLD, rather than endogenous cervical cell PLD, in the observed increase in PLD activity described above.

10 Analysis of PLD activity in infected and uninfected cervical cell fractions revealed a significant portion of gonococcal and cervical cell PLD activity lies within the membrane-enriched portion of cervical cell lysates. However, PLD activity was also observed in the cytosolic-enriched cell fraction. No significant difference was observed between PLD activity in uninfected cervical cells and the

15 PLD mutant infected cells.

Membrane ruffling followed by macropinocytosis of gonococci serves as a primary mechanism by which these bacteria invade the cervical epithelium. To determine if NgPLD non-specifically gains access to the cervical cell cytosol during macropinocytosis of gonococci, cell fractionation studies were performed of infected

20 and uninfected pex cells treated or untreated with wortmannin or cytochalasin D (Table 14). PLD activity was significantly reduced in membrane- and cytosol-enriched cell fractions when wortmannin and cytochalasin D were included in wild-type infection studies. No significant difference was observed in PLD activity in uninfected or 1291ΔPLD infected pex cells when these same cytoskeletal

25 inhibitors were included or excluded from the assay. Collectively, these data indicate that macropinocytosis of invasive gonococci allows NgPLD to enter primary cervical cells.

Table 14. PLD activity in cervical cells treated with cytoskeletal inhibitors.

PLD Activity in Ectocervical Cell Fractions				
Fluorescence Units [Mean (variance)]				
	+/- Wortmannin Treatment		+/- Cytochalasin D Treatment	
5	Cell Fraction	Membrane	Cytosolic	Membrane Cytosolic
	Uninfected w/o	0.578 (0.04)	0.388 (0.02)	0.368 (0.04) 0.479 (0.05)
	Uninfected w/	0.474 (0.05)	0.403 (0.01)	0.389 (0.01) 0.395 (0.03)
	WT 1291 w/o	0.992 (0.10)	0.863(0.08)	1.035 (0.06) 0.846 (0.09)
10	WT 1291 w/	0.784 (0.06)	0.413 (0.03)	0.436 (0.03) 0.462 ((0.03)
	1291ΔPLD w/o	0.410 (0.01)	0.406 (0.02)	0.343 (0.01) 0.392 (0.01)
	1291ΔPLD w/	0.418 (0.02)	0.408 (0.01)	0.393 (0.01) 0.330 (0.07)
Assay Controls				
15	Positive Control	1.133 (0.29)	Negative control	0.180 (0.04)

Cellular fractionation and PLD activity were assayed as described in the text. Data given are the mean values obtained from three trials in which gonococci were removed by filtration through a 0.22 μm syringe filter (see materials and methods).

- 20 **Gonococcal PLD Acts at Multiple Levels in Cervical Cell Infection.** Studies using *Streptomyces* PLD have indicated that the exogenous addition of PLD to vascular smooth muscle cells mimics endogenous PLD activity within these cells (Kondo et al., 1992). Consequently, it was determined if exogenously added PLD substrates could compete with cervical cell constituents for NgPLD activity and in
- 25 doing so interfere with the role of NgPLD in gonococcal invasion. The addition of 10, 1, 0.1, or 0.01 μg/ml of phosphatidylcholine (PtC) or 1.0, 0.1, or 0.01 percent ethanol to infection studies impaired the ability of gonococci to invade pex cells in a dose-dependent manner (Table 15). In contrast, no effect was observed in

gonococcal association with and/or their invasion of primary cervical cells in the presence of 1.0, 0.1, or 0.01 percent 2-butanol (Table 15), which can not serve as a substrate in PLD-catalyzed transphosphatidylation. There was no significant difference in survival observed between gonococci incubated in the presence or absence of 1.0, 0.1, or 0.01 percent ethanol or 2-butanol in the absence of cervical cells (data not shown). These data suggest a role for gonococcal PLD in modulating cervical cell signaling events (*e.g.*, through phosphatidic acid (PA) generation) and suggest that NgPLD may function at several different levels in gonococcal invasion of cervical epithelia.

10

Table 15

Percent adherence to and/or invasion of primary cervical cells by gonococci in the presence or absence of PLD substrate competitors

A.		<b>1291 WT</b>		<b>1291ΔPLD</b>
	<b>Competimer</b>	<b>Inhibition<sup>1</sup></b> (%)	<b>Invasion<sup>1</sup></b> (%)	<b>Invasion<sup>2</sup></b> (%)
15	<b>None</b>	NA	2.7833 (0.16)	0.3371 (0.02)
	<b>Ethanol (%)</b>			
	<b>1.0</b>	90.7346 (0.92) p<0.05	0.2564 (0.01) p<0.05	0.3288 (0.03) p>0.75
	<b>0.1</b>	80.9243 (1.41) p<0.05	0.5288 (0.01) p<0.05	0.3419 (0.04) p>0.90
	<b>0.01</b>	74.9582 (0.87) p<0.05	0.6957 (0.02) p<0.05	0.3360 (0.04) p>0.90
20	<b>2-Butanol (%)</b>			
	<b>1.0</b>	ND	2.6869 (0.47) p>0.90	0.2960 (0.05) p>0.25
	<b>0.1</b>	ND	2.7087 (0.47) p>0.75	0.3172 (0.03) p>0.25

<b>0.01</b>	ND	2.8661 (0.53) p>0.75	0.2819 (0.02) p>0.25
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B.

	<b>PtC ( g/ml)</b>	<b>Inhibition<sup>1</sup> (%)</b>	<b>Invasion<sup>1</sup> (%)</b>
5	<b>10.0</b>	98.7786 (0.08) p<0.05	0.0331 (0.01) p<0.05
	<b>1.0</b>	92.4681 (0.38) p<0.05	0.2038 (0.01) p<0.05
	<b>0.1</b>	87.3571 (0.28) p<0.05	0.3418 (0.01) p<0.05
	<b>0.01</b>	68.8105 (2.15) p<0.05	0.8379 (0.03) p<0.05
	<b>0.0</b>	NA	2.7052 (0.11) p<0.05

- 10 Values given are the mean values in which the percent invasion was determined as a function of the original inoculum and the subsequent number of colony forming units formed with subsequent plating of the ecto- or endocervical cell lysates. Inhibition values given were determined as a normalized function of the ability of the gonococcus to invade primary ectocervical cells in the presence of, in
- 15 comparison to the absence of, an alcohol and phosphatidylcholine competimers as outlined in the text. Data given are the mean values obtained from at least three trials performed in triplicate. Variances are noted parenthetically. *P*-values were determined using a Kruskal-Wallis k-sample analysis of variance.

NA - Not applicable

- 20 ND - Not determined

1 - *p*-values were calculated for wild-type gonococci in the presence of competimer compared to the absence of the competimer

2 - *p*-values were calculated for PLD mutant gonococci in the presence of competimer as compared to the absence of the competimer

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## Discussion

Phospholipases are a diverse group of hydrolytic enzymes, which are classified by the specificity they exert for the site of phospholipid cleavage. In eukaryotic systems, homologs of PLD can be activated by a variety of stimuli (*e.g.*, hormones and growth factors) after which they catalyze the hydrolysis of PtC to choline (Cho) and PA (Exton, 1997; Jones et al., 1999; Waite, 1999). PLDs belong to a large superfamily of proteins, which can be divided into eight classes (Ponting and Kerr, 1996). Included in the PLD superfamily are prokaryotic and eukaryotic PLDs, cardiolipin and phosphatidylserine synthases, Vaccinia and Fowlpox viral proteins of unknown function, an *Escherichia coli* nuclease and an *E. coli* helicase (Ponting and Kerr, 1996). All members of the PLD superfamily contain (usually) two HKD motifs, which are thought to associate to form a catalytic center. However, unique to PLD is the ability to catalyze a transphosphatidylolation reaction in which a primary alcohol preferentially serves as a nucleophilic acceptor instead of water, resulting in the near-exclusive production of a phosphatidylalcohol (PtOH) at the expense of PA. The resulting PtOH is metabolically stable and, thus, serves as a specific indicator of PLD activity. Although eukaryotic PLDs have been well studied, much less is known about bacterial PLDs, and, in fact, only a handful have been identified. Some bacterial PLDs are associated with virulence, *e.g.*, the Yersinia murine toxin (Ymt) (Hinnebusch et al., 2002) and PLDs of *Corynebacterium spp.* (McNamara et al., 1995), pathogens of humans and domestic animals. It is demonstrated in *Corynebacterium pseudotuberculosis* that PLD mutation results in the attenuation of this microbe (Hodgson et al., 1992; Simmons et al., 1998).

PLD activity in *N. gonorrhoeae* is disclosed herein, as well as a role for this secreted protein in gonococcal pathogenesis of cervical epithelia. Characteristic PLD activity (*i.e.*, removal of Cho by cleavage of the terminal phosphodiester bond of PtC) was observed in gonococcal whole cell lysates but was absent in gonococci in which *pld* was mutated by the insertion of a kan<sup>R</sup> cassette. PLD activity was

observed over a pH range of 3.0 to 7.4, which is consistent with its ability to function as an effector protein within the lower female genital tract under normal (uninfected) or diseased states. The ability of NgPLD activity to promote gonococcal invasion of primary cervical cells was inhibited in the presence of PtC and ethanol (a primary alcohol) but not 2-butanol (a secondary alcohol). These data definitively demonstrate that this gonococcal protein does indeed exhibit characteristic PLD function and argue against a role for endogenous pex or pen cell phospholipase C (PLC) activity in CR3-mediated invasion of cervical epithelia by gonococci. A BLAST search of the *N. gonorrhoeae* and *N. meningitidis* genomic databases using sequences to several bacterial PLCs failed to reveal the presence of this enzyme in the pathogenic *Neisseria*. Furthermore, these data indicate that the generation of PA or its catabolic products are required for CR3-mediated macropinocytosis of gonococci.

Recent evidence indicates that Ymt promotes the survival of *Y. pestis* within the flea midgut from a cytotoxic digestion product present in blood plasma and, consequently, promotes disease transmission (Hinnebusch et al., 2002). Data herein indicate that the presence of a functional NgPLD is essential for the survival of gonococci within primary cervical cells. Although an interaction with the ASGP-R does not appear to sustain NgPLD secretion in culture supernatants, NgPLD does play a role in gonococcal survival within urethral epithelial cells. The addition of SsPLD to association and invasion assays of *N. gonorrhoeae*  $\Delta$ PLD infected cervical cells did not compensate for the absence of NgPLD, suggesting NgPLD exhibits unique effector functions in addition to sharing structural and functional properties with SsPLD. This is supported by the finding that, although all PLDs contain (usually) two HKD motifs, sequences outside these regions are not necessarily highly conserved and may confer specific effector functions to their respective proteins (Waite, 1999).

Total PLD activity was greater in infected pex and pen cells upon comparison to uninfected or PLD mutant infected cells, which was attributed to

NgPLD and not endogenous PLD activity. NgPLD appears to modulate cervical cell function, either directly or indirectly in a cooperative manner with host cell effector molecules, to promote the appropriate targeting of gonococci to permissive host cells (*i.e.*, CR3-expressing ecto- and endocervical cells) and to ensure their  
5 intracellular survival.

The association with and invasion of primary cervical epithelia is impaired in the absence of NgPLD. CR3, the primary receptor by which gonococci invade the cervical epithelia, is not recruited to the cervical cell surface in the absence of NgPLD. Membrane ruffling is not evident in the absence of NgPLD with extended  
10 infection. Thus, NgPLD is unique among prokaryotic proteins identified to date.

The ability of PLD to cause the release of secondary granules in neutrophils suggests that this molecule may play a role in the recruitment of CR3 to the surface of these cells. Additionally, products of PLD-catalyzed phospholipid hydrolysis serve as second messengers, eliciting a variety of cellular responses and are thought  
15 to function in complement (C')-mediated endocytosis (Fällman et al., 1992) and in cytoskeletal rearrangements (Colley et al., 1997; Ha and Exton, 1993; Jones et al., 1999). The absence of CR3 recruitment to the cell surface in *N. gonorrhoeae*  $\Delta$ PLD infected primary cervical cells strongly suggests an early role for NgPLD in, directly or indirectly, modulating CR3 effector function. Studies using *Streptomyces*  
20 *chromofuscus* PLD (ScPLD) indicate that exogenous ScPLD can mimic endogenous PLD activity by triggering cytoskeletal rearrangements, DNA synthesis, and cell proliferation (van Dijk et al., 1998; Kondo et al., 1992). These data provide a precedent for the observations herein demonstrating the ability of exogenous NgPLD, a secreted bacterial protein, to modulate CR3 effector function in primary  
25 cervical epithelial cells.

Reorganization of the actin cytoskeleton is the result of the activation of a complex network of signal transduction pathways involving many effector molecules. Bacterial, plant, and human PLDs directly bind polymeric F-actin, which in turn increases PLD activity (Kusner et al., 2003). In contrast, monomeric G-actin



inhibits PLD activity in a species-specific manner in that (Kusner et al., 2002), *in vitro*, G-actin-induced PLD inhibition is twenty-fold greater for human PLD1 than it is for SsPLD (Kusner et al., 2003). The greatest degree of inhibition occurs upon the initiation of PLD activity in the presence of G-actin; less inhibition is observed  
5 when G-actin is added to previously activated PLD (Kusner et al., 2003). Phosphatidylinositol 4, 5-bisphosphate (PIP<sub>2</sub>) is a required co-factor in human PLD activity; in contrast, bacterial PLD activity does not exhibit a cofactor requirement. In resting cells, mammalian PLD resides in an inactive state because PIP<sub>2</sub>, which remains bound to actin-associated proteins (*e.g.*, vinculin, -actinin, fodrin), is  
10 unavailable as a required co-factor (Lukowski et al., 1996).

Cervical cells infected with the *N. gonorrhoeae* ΔPLD mutant failed to elicit membrane ruffling but did promote microvilli/filopodia formation, suggesting NgPLD might be required to potentiate the extensive cytoskeletal rearrangements necessary for ruffle formation. NgPLD may act in a synergistic or an additive  
15 manner with endogenous cervical cell PLD to potentiate membrane ruffling by stabilizing actin filaments in a manner similar to what is observed with phalloidin.

Disclosed herein are studies that elucidate the signaling pathways that participate in the response of cervical epithelia to *N. gonorrhoeae* infection. In this respect, a novel gonococcal virulence factor, NgPLD, which modulates CR3 effector  
20 function in conjunction with cervical cell effector molecules to trigger alternative signal transduction pathways is identified. This secreted gonococcal product serves a critical role in ensuring appropriate targeting of the gonococcus to the ecto-and endocervical epithelium by recruiting CR3 to the cervical cell surface and promotes intracellular survival of gonococci following CR3-mediated macropinocytosis.

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All publications, patents and patent documents are incorporated by reference herein, as though individually incorporated by reference. The invention has been described with reference to various specific and preferred embodiments and

techniques. However, it should be understood that many variations and modifications may be made while remaining within the scope of the invention.

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